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Chi-Hua Nieh

2013

Table of Contents

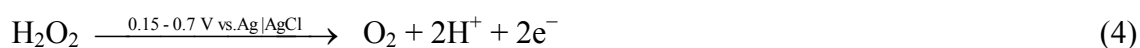
General Introduction.....	1
Chapter 1 Electrostatic and steric interaction between redox polymers and some flavoenzymes in mediated bioelectrocatalysis.....	7
Introduction.....	7
Experimental.....	11
Results and Discussion	14
Reference	23
Chapter 2 Four enzyme-based biosensor mediated by PVI[Fe(CN)₅] for creatinine determination.....	25
Introduction.....	25
Experimental.....	28
Results and Discussion	30
Reference	40
Chapter 3 Sensitive D-amino acid bienzyme biosensor mediated by PVI[Fe(CN)₅]	41
Introduction.....	41
Experimental.....	43
Results and Discussion	45
Reference	56
Conclusions	57
Appendix	59
Acknowledgement.....	63
List of publications	64

General Introduction

H₂O₂-producing oxidase and peroxidase (POD)-coupled system has been widely utilized for analyte detection by the spectrophotometric method, since POD reacts with several kinds of reduced reagents that can be oxidized into fluorescent or colored product for detection, thanks to poor substrate specificity of POD [1-3]. However due to the property, the POD system is labile to cause negative interference by oxidizing electroactive compounds such as ascorbic acid (AA) or uric acid in physiological fluids, especially in solution reactions (Eq. 1) [4]. The reaction of the interference with H₂O₂ catalyzed by POD decreases the H₂O₂ concentration to cause the underestimation of the H₂O₂ concentration.



To solve the problem, electrochemical biosensors with immobilized H₂O₂-producing oxidases covered with membrane have been developed with the advantages of low cost, rapid and simple operation, since Clark and Lyons proposed an amperometric enzyme-immobilized electrode for glucose detection in 1962 [5]. In enzyme-based amperometric biosensors, the concentration of analyte can be directly determined based on the detection of oxygen consumption (Eqs. 2 and 3) or generated H₂O₂ (Eq. 4). The detection system is so-called the first generation biosensor [6, 7].

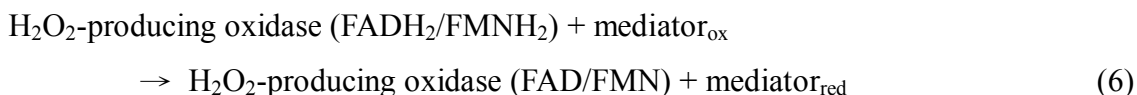
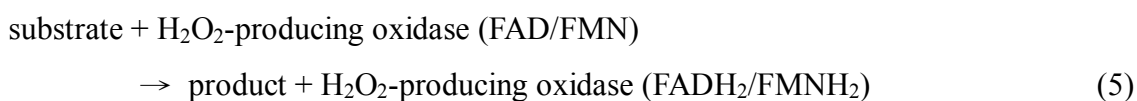


In addition, the usage of membrane on the top of immobilized enzyme layer can eliminate the interference effect based on perm-selectivity [8]. Interferences can be

blocked based on size exclusion or electrostatic repulsion. The interference effect due to the poor specificity of POD which exists in the aforementioned spectrophotometric method can be overcome.

However, in the detection of oxygen consumption based on the reduction of oxygen, the signal response is seriously influenced by the concentration of dissolved oxygen in samples and the diffusion rate of oxygen from the bulk solution to the surface of working electrode. On the other hand, the direct electrooxidation of H_2O_2 requires high operating potential (+0.7 V vs. Ag|AgCl); although membrane can exclude interferences, for the purpose of high sensitivity, sometimes membrane is insufficient since the high operating potentials lead to oxidation of the small amount of electroactive compounds in samples which may pass through the membrane to cause serious interference problem.

In order to overcome this problem, the second generation biosensors have been evolved by using mediators to regenerate oxidases (Eqs.5 and 6) [5, 9-11],



where FAD is flavin adenine dinucleotide and FMN is flavin mononucleotide. This type of biosensor has been evolved by using redox substances (mediators) such as DCPIP and benzoquinone to shuttle electrons between the redox center of enzyme and electrode, which provides higher signal response and allows lower operating potential.

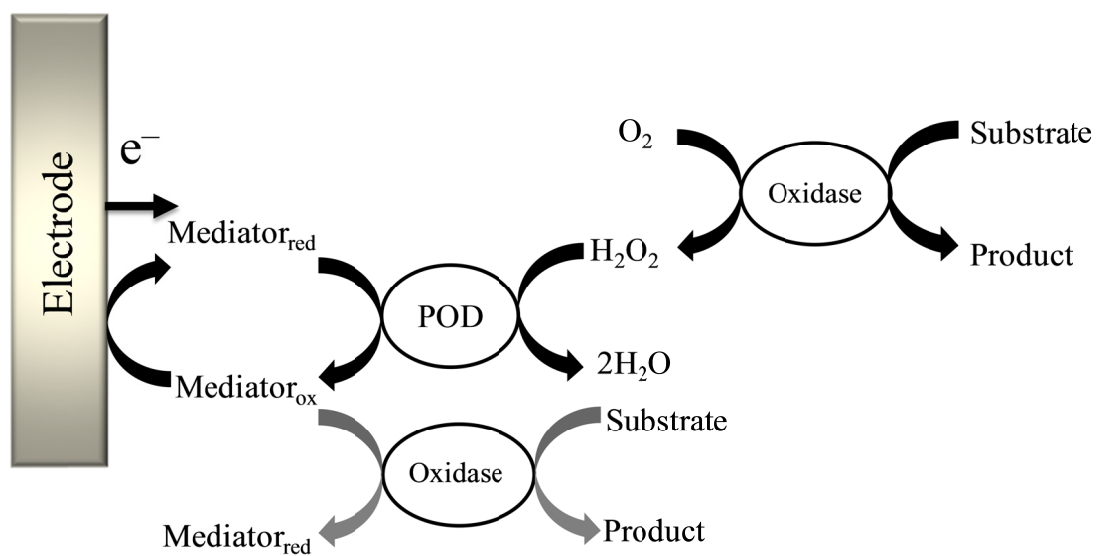
Nevertheless, it is still hard for the second generation biosensors to satisfy the need of high sensitivity since the operation at positive potentials (0 – 0.5 V vs. Ag|AgCl) leads to an increase in oxidative interferences in physiological samples (e.g., the oxidation potential of AA is ca. 0 V vs. Ag|AgCl); furthermore, dissolved oxygen needs to be removed to avoid the competition with the mediator, which is difficult in practical analysis.

To circumvent the problems, H₂O₂-producing oxidase/POD-based biosensors have been proposed, in which H₂O₂ generated from an oxidase reaction is reductively detected at low operating potentials around 0 V vs. Ag|AgCl with the aid of POD in the mediated-electron-transfer system [12-14]. Since the low potential operation and the usage of membrane decrease the background current and noise levels, furthermore eliminate the undesirable oxidation of electroactive interference, bienzyme biosensors show high sensitivity and stability.

Nevertheless, the other thing which needs to be concerned is that the mediators may react with both of oxidase and POD. The mediator oxidized by POD may be reduced not only at electrode but by oxidase, because most of oxidases show dehydrogenase activity to transfer electrons to artificial mediators (Scheme 1) [15, 16]. Such cross reaction diminishes the cathodic current to cause a decrease in the electrochemical response of mediator reduction.

In consideration of 1) the possibility of cross reaction of mediators with POD and oxidases, 2) the interference effect of O₂ reduction around -0.2 V (vs. Ag|AgCl), and 3) the occurrence of interference oxidation at positive potentials, it becomes an important issue for mediated bienzyme biosensors to select an appropriate mediator with high selectivity for POD alone and a suitable operating potential in the narrow range from -0.2 to 0 V.

The author in this research attempted to find a suitable mediator with selective reactivity against POD alone for such oxidase/POD-based biosensors, and constructed two kinds of biosensors for practical purpose.



Scheme 1 The electron transfer profile of oxidase/POD bienzyme sensor system. The gray-colored arrows indicate the electron transfer path in negative interference due to dehydrogenase activity of oxidase.

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Chapter 1 Electrostatic and steric interaction between redox polymers

and some flavoenzymes in mediated bioelectrocatalysis

H₂O₂-producing oxidase/peroxidase (POD)-based mediated biosensors are very useful to minimize interference, but require suitable mediators which work well only for POD but not against the oxidase. Pentacyanoferrate-bound poly(1-vinylimidazole) (PVI[Fe(CN)₅]), PVI[Os(dcbppy)₂Cl] (dcbppy = 4,4'-dicarboxy-2,2'-bipyridine) and PVI[Os(dmebpy)₂Cl] (dmebpy = 4,4'-dimethyl-2,2'-bipyridine) have been utilized to investigate the interaction with four kinds of H₂O₂-producing oxidases: glucose oxidase, sarcosine oxidase, choline oxidase (ChOD) and lactate oxidase. The mediated bioelectrocatalytic activities of the redox polymers for the enzymes have been determined by cyclic voltammetry in the presence of the substrates. The highly negatively charged PVI[Fe(CN)₅] shows practically no mediating activity against the four flavoenzymes, but strong one to POD. On the other hand, PVI[Os(dmebpy)₂Cl] with neutral ligands shows a high activity for the oxidases except ChOD. The mediating activity of PVI[Os(dcbppy)₂Cl] with negatively charged ligands is much smaller than that of PVI[Os(dmebpy)₂Cl]. These results reveal that electrostatic repulsion and steric hindrance are enhanced by using negatively charged polymers to realize minimum activity against the oxidases.

Introduction

Various types of transition metal redox polymers such as Os and Ru complexes and ferrocene derivatives have been developed for co-immobilization of enzymes to construct mediated electron transfer (MET) bioelectrocatalytic systems over the past two decades [1-3]. These electroconductive polymers are conspicuous due to their unique properties over diffusional mediators: they can be covalently bound to enzymes by crosslinkers on electrode surface; they provide three-dimensional electrocatalytic systems which are not leachable but swollen in water to form redox hydrogels for MET

between the redox center of enzymes and electrodes; they have so high density of the redox groups immobilized on electrode surface that they are more efficient than low molecular weight mediators in the electron transfer; moreover, transition metal redox polymers are more stable than quinone-containing polymers since reactive semiquinone radicals formed in the one-electron reduction of quinones easily react with thiols, amines, phenols and other functions [4-6]. These unique properties of metal redox polymers are very useful for MET-type biosensor and biofuel cell application [5, 7, 8]. In MET glucose biosensors, Os-containing polymers have frequently been utilized to shuttle electrons from the redox center of glucose oxidase (GOD) or flavin adenine dinucleotide (FAD)-dependent glucose dehydrogenase (GDH) to electrode, and show higher stability and sensitivity than low molecular weight mediators [1, 9]. In MET bioelectrocatalysis, we have to consider the linear free energy relationships (LFER) of the electron transfer rate constant (k) between enzyme and mediator to the formal potential of mediator (E°) [10-12]. The relation is given by Eq. (1.1) for the oxidation of the substrate,

$$\log \frac{k_j}{k_i} = \beta \frac{nF}{2.303RT} (E^\circ_j - E^\circ_i) \quad (1.1)$$

where β is a proportional constant ($0 < \beta < 1$), n is the number of electrons, F is the Faraday constant, R is the gas constant, T is absolute temperature, and the subscripts i, j indicate given mediators as a series of redox compounds with similar structure. In order to increase the current density, it is essential to use a mediator with a large k value and then with a more positive E° value, which leads to increase the oxidative interferences such as ascorbic acid and uric acid in physiological samples. Furthermore, as mentioned in *General Introduction*, O_2 must sometimes be removed to avoid the competition with mediators in the case of oxidase, which would be difficult and not practical in real sample measurements.

In order to solve the problems, H_2O_2 -producing oxidase/peroxidase (POD) bienzyme biosensors mediated by Os-containing polymers were developed [13]. Oxidase-based mediated biosensors coupled with POD allow the determination of H_2O_2

generated from the oxidase such as GOD at low operating potentials around 0 V vs. Ag|AgCl, with high sensitivity and stability, and the elimination of the undesirable oxidation of interferences [14, 15]. However, as mentioned in *General Introduction*, mediators may react with both of oxidase and POD. The oxidized mediators may also act as electron acceptors of H₂O₂-producing oxidases based on the dehydrogenase activity of the oxidase to cause a decrease in the electrochemical response of mediator reduction. Therefore, it is necessary to select an appropriate mediator with highly selective reactivity for POD but practically no reactivity for oxidase.

The k value in MET bioelectrocatalysis also depends on the structure and charge of the mediator. In the case of PQQ-dependent GDH, the k values of Os-complexes are much lower than those of quinone compounds at a given E° [12]. The main factor to cause the difference seems to be the steric effect, since the size of the Os-complex is much larger than that of the active site of the enzyme. In the case of GOD, the k values of negatively charged inorganic and organic mediators are much lower than those of neutral quinone mediators with almost identical E° [16]. The electrostatic repulsion between negatively charged mediators and the active site of GOD is expected.

The author expects in this work that such steric hindrance and electrostatic repulsion will be enhanced by using redox polymers with large molecular weight and high density of negative charge. On the other hand, such negative effects seem to be minimized for POD, because the catalytic center of POD is located on the surface of the enzyme and the vicinity of the catalytic center is positively charged [17].

In order to verify the hypothesis, the author focuses on pentacyanoferrate-bound poly(1-vinylimidazole) (PVI[Fe(CN)₅]) (Fig. 1-1A) as a redox polymer mediator for oxidase/POD bienzyme biosensors. The mediator has been synthesized for high MET activity with bilirubin oxidase (BOD) [18]. Os-complex-bound PVIs, PVI[Os(dmebpy)₂Cl] (dmebpy = 4,4'-dimethyl-2,2'-bipyridine) with neutral ligands (Fig. 1-1B) and PVI[Os(dcbppy)₂Cl] (dcbppy = 4,4'-dicarboxy-2,2'-bipyridine) with negatively charged ligands (Fig. 1-1C) are also used as references.

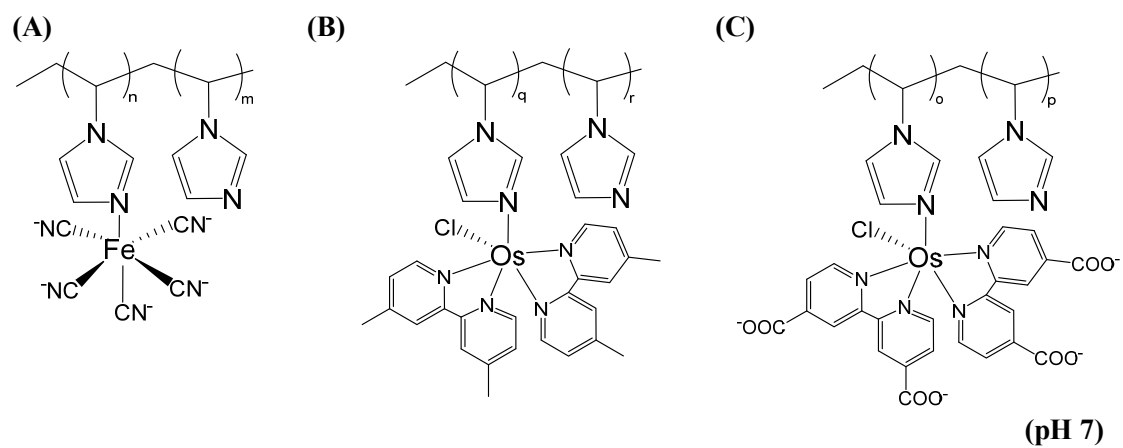


Figure 1-1 Structures of (A) PVI[Fe(CN)₅], (B) PVI[Os(dmebpy)₂Cl] and (C) PVI[Os(dcbppy)₂Cl].

Experimental

Reagents

(NH₄)₂[OsCl₆], 4,4'-dimethyl-2,2'-bipyridine, and 1-vinylimidazole were purchased from Sigma-Aldrich Co. (USA). 2,2'-Bipyridine-4,4'-dicarboxylic acid, ethylene glycol, sodium hydrosulfite (Na₂S₂O₄), 2,2'-azobisisobutyronitrile (AIBN), diethyl ether, sodium pentacyanonitrosylferrate(III) dihydrate (Na₂[Fe(CN)₅(NO)]·2H₂O), glucose, sarcosine, choline chloride and L-lactate were obtained from Wako Pure Chemical Industries (Osaka, Japan). POD from horseradish (POD, 257 U mg⁻¹), GOD from *Aspergillus* sp. (100 U mg⁻¹), sarcosine oxidase from microorganism (SOD, 16.6 U mg⁻¹), choline oxidase from *Alcaligenes* sp. (ChOD, 16.9 U mg⁻¹) and lactate oxidase from microorganism (LOD, 101 U mg⁻¹) were from Toyobo Co. (Osaka, Japan). Substrate solutions and other enzyme solutions were prepared with a phosphate buffer solution (100 mM, pH 7.0). 1 M Na₂S₂O₄ was prepared with distilled water. Other chemicals were of analytical grade and used as received.

Synthesis of mediator-containing polymers

Os(dmebpy)₂Cl₂ and Os(dcbppy)₂Cl₂ were synthesized as reported [19]. In brief, (NH₄)₂[OsCl₆] (0.57 mmol) and 2 equivalents of 4,4'-dimethyl-2,2'-bipyridine or 2,2'-bipyridine-4,4'-dicarboxylic acid were dissolved in 9 mL of ethylene glycol, heated under reflux and stirring for 2 h in Ar. After cooling to room temperature, the solution was treated with 15 mL of 1 M Na₂S₂O₄ to reduce [Os(dmebpy)₂Cl₂]⁺ or [Os(dcbppy)₂Cl₂]⁺ which might be formed during the synthesis, and then cooled in an ice bath for 30 min. The dark-violet precipitate was obtained after being washed with distilled water and diethyl ether.

Poly(1-vinylimidazole) (PVI) was prepared according to the literature [1]. Briefly, 6 mL of 1-vinylimidazole mixed with 0.5 g of AIBN which works as an initiator was heated at 70 °C for 2 h under Ar with stirring. After cooling, the yellow precipitate was observed and re-dissolved with methanol, followed by adding the methanol solution

dropwise to acetone under strong stirring. White PVI powder was obtained after filtering and drying.

PVI[Os(dmebpy)₂Cl] and PVI[Os(dcbppy)₂Cl] were also prepared according to the literature [1]. The powder of synthesized Os(dmebpy)₂Cl₂ (66 mg, ca. 0.105 mmol) or Os(dcbppy)₂Cl₂ (85 mg, ca. 0.105 mmol) and PVI (100 mg, ca. 1.05 mmol) were dissolved in 100 mL of absolute ethanol to avoid the replacement of second chloride ion in Os complex in the presence of H₂O during the following step of heating. The mixture was heated at reflux and stirred for 3 days. The precipitate was obtained by adding the solution to diethyl ether under stirring. After filtering and drying, the precipitate was dissolved in 10 mL of the phosphate buffer solution (10 mM, pH 7.0) and stored at 4 °C. The final concentration of the two polymers was about 18 mg mL⁻¹. The ratio of the imidazole unit in PVI to the Os(dmebpy)₂Cl complex may be approximately 10 according to the literature [1].

PVI[Fe(CN)₅] was synthesized similar to the method in the literature [18]. In brief, 200 mg of Na₂[Fe(CN)₅(NO)]·2H₂O and 188 mg of PVI were dissolved in 50 mL of 0.6 M NaOH and were refluxed at 65 °C for 24 h. During the process, NO⁺ was released through the reaction with OH⁻, and the pentacyanoferrate ion was linked to N in imidazole. The mixture was afterward dialyzed against distilled water for 24 h to remove unreacted compounds. After centrifuged at 5000 g for 20 min 2 times to remove red precipitate, the suspension was vacuum freeze-dried at -40 °C for 24 h to get PVI[Fe(CN)₅] powder. The ratio of the imidazole unit in PVI to the Fe(CN)₅ complex was 4.3 as measured by elemental analysis. The stock solution of PVI[Fe(CN)₅] was prepared by dissolving in 10 mM phosphate buffer at pH 7.0.

Electrochemical measurements

Electrochemical measurements were performed with glassy carbon electrodes (3 mm, BAS) and carried out in the phosphate buffer solution (100 mM pH 7.0) at 25 °C with an electrochemical analyzer (BAS CV 50 W, BAS Inc., Japan). All potentials are referred to Ag|AgCl|sat. KCl reference electrode in this work. Considering the fact that the carboxyl group of PVI[Os(dcbppy)₂Cl] is also crosslinked with poly(ethylene glycol) diglycidyl ether, the redox polymers were used in soluble state without immobilization on electrode surface. The final concentration of the Os-containing polymers in the

sample solution was one tenth of the Os-containing polymer stock solutions, while the final concentration of PVI[Fe(CN)₅] was 0.3 mg mL⁻¹ in the sample solution. The peak anodic currents of the diluted PVI[Os(dmebpy)₂Cl] and PVI[Os(dcbppy)₂Cl] solutions were, respectively, 260 and 350 nA at a scan rate of 20 mV s⁻¹ in cyclic voltammetry. Supposing that the diffusion coefficients of the two Os-containing polymers are close to each other, the concentrations of Os^{2+/3+} in the sample solutions were in the same level. The activity of the metal redox polymer against enzyme was also evaluated by cyclic voltammetry.

Measurements of enzyme's concentration

The concentrations of GOD, SOD and LOD were determined spectrophotometrically. The molar extinction coefficients of GOD, SOD and LOD were chosen as 13.0 mM⁻¹ cm⁻¹ at 450 nm [20], 12.2 mM⁻¹ cm⁻¹ at 454 nm [21] and 12.5 mM⁻¹ cm⁻¹ at 450 nm [22], respectively.

Results and Discussion

The formal potentials ($E^{\circ'}$) of PVI[Os(dmebpy)₂Cl], PVI[Os(dcbppy)₂Cl] and PVI[Fe(CN)₅] were determined, respectively, as 0.156 V, 0.204 V and 0.213 V by cyclic voltammetry. Their MET activities against GOD are shown in Fig. 1-2. The clear catalytic oxidation current of glucose was observed with PVI[Os(dmebpy)₂Cl] after addition of GOD (Fig. 1-2A), while the catalytic current with PVI[Os(dcbppy)₂Cl] was much smaller than that with PVI[Os(dmebpy)₂Cl] (Fig. 1-2B). The MET activity can be quantitatively expressed by the bi-molecular reaction rate constant between enzyme and mediator, k_{cat}/K_M , where k_{cat} is the catalytic constant of the enzyme and K_M is the Michaelis constant for the mediator. The k_{cat}/K_M can be easily evaluated from the slope of the linear relation between the limited catalytic current (I_c) and the total concentration of mediator ($[M]$) at $[M] < K_M$ [23],

$$I_c = n_M F A [M] \sqrt{(n_S / n_M) D_M k_{\text{cat}} [E] / K_M} \quad (1.2)$$

where n_M and n_S are the number of electron of mediator and substrate, respectively, A is the electrode surface area, D_M is the diffusion coefficient of mediator, and $[E]$ is the concentration of enzyme. In this work, the $\sqrt{D_M [M]}$ values for the redox polymers were evaluated from the peak current (I_p) of cyclic voltammetry of the polymer solution in the absence of enzyme by assuming the reversible response of the mediator at a given scan rate (v) [24].

$$I_p = -0.4463 n_M F A [M] \sqrt{n_M F v D_M / RT} \quad (\text{at } 25^\circ\text{C}) \quad (1.3)$$

The k_{cat}/K_M value was calculated from the $(I_c/I_p)^2$ at a given concentration of mediator,

$$k_{\text{cat}} / K_M = (I_c / 3.59 I_p \sqrt{[E]})^2 \quad (1.4)$$

at $n_M = 1$, $n_S = 2$, and $\nu = 0.02 \text{ V s}^{-1}$ (for the experimental conditions). The result is summarized in Table 1-1, which indicates that the MET activity of PVI[Os(dmebpy)₂Cl] is approximately one order larger than that of PVI[Os(dcbppy)₂Cl]. Since the E° and the size of the two Os-containing polymers are almost identical with each other, the large difference in the MET activity cannot be explained in terms of LFER, but some electrostatic effect is expected.

The active site of GOD, FAD, is in hydrophobic surroundings and is buried in the molecule [25]. Moreover, GOD has essentially negative electrostatic surface potential at pH 7 and the surface electrostatic potential of the channel to FAD is negative (Fig. 1-3) [26, 27]. Therefore, PVI[Os(dcbppy)₂Cl] with the negatively charged ligand ($-\text{COO}^-$) is more difficult to reach the FAD center in GOD than PVI[Os(dmebpy)₂Cl] with the neutral ligand due to the repulsive electrostatic interaction. The results are consistent with the previous research that the rate constant of the electron transfer between GOD and Os-complexes is strongly related to the charge of the Os complexes: Os(dmebpy)₂(pyNH₃⁺)(imNH₃⁺) (global charge (the net charge of Os³⁺ and ligands) = +5, py = pyridine, im = imidazole) > Os(dmebpy)₂(py)(imNH₃⁺) (global charge = +4) > Os(dmebpy)₂Cl(pyNH₃⁺) (global charge = +3) [28], indicating the attractive electrostatic interaction between positively charged Os-complex and GOD [29, 30].

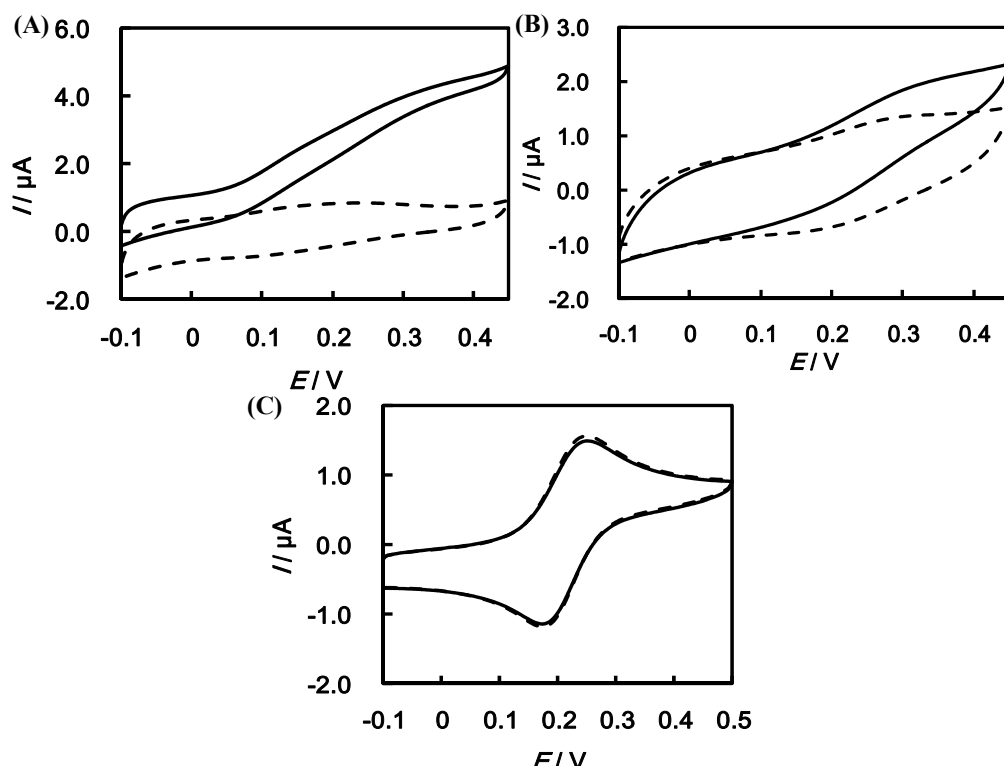


Figure 1-2 Cyclic voltammograms of 500 mM glucose solution containing (A) PVI[Os(dmebpy)₂Cl], (B) PVI[Os(dcbppy)₂Cl], and (C) PVI[Fe(CN)₅] in the absence (dashed line) and presence (solid line) of GOD (39.2 U mL⁻¹) at a scan rate of 20 mV s⁻¹.

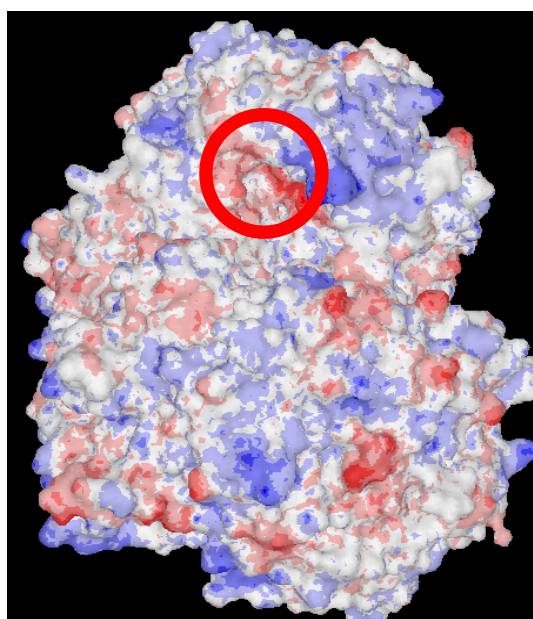


Figure 1-3 Surface electrostatic potential of GOD dimer. The circle indicates one of the positions of FAD. Red: negative potential, Blue: positive potential. (PDB: 1GPE)

When PVI[Fe(CN)₅] was used as a mediator, no clear catalytic current was observed in the GOD system (Fig. 1-2C), in spite of the fact that the size of PVI[Fe(CN)₅] ($7.2 \times 6.2 \text{ \AA}^2$) is approximately three times smaller than that of PVI[Os(dcbppy)₂Cl] ($11.2 \times 13.8 \text{ \AA}^2$) (Fig. 1-4) and the E° is almost identical with that of PVI[Os(dcbppy)₂Cl] (Table 1-1). The extremely low MET activity of PVI[Fe(CN)₅] against GOD can be interpreted by the increased repulsive electrostatic interaction between PVI[Fe(CN)₅] and GOD, since the negative charge density of PVI[Fe(CN)₅] is much higher than that of PVI[Os(dcbppy)₂Cl].

It is well known that hexacyanoferrate ion is frequently utilized as a mediator of GOD-based MET-type glucose biosensor [31-33], although the MET activity is low [16]. The fact and the present result indicate that the polymerization of pentacyanoferrate increases repulsive electrostatic effect. The polymerization also seems to introduce the steric hindrance effect. As a result, the author has successfully found a redox polymer (PVI[Fe(CN)₅]) with practically no MET activity against GOD.

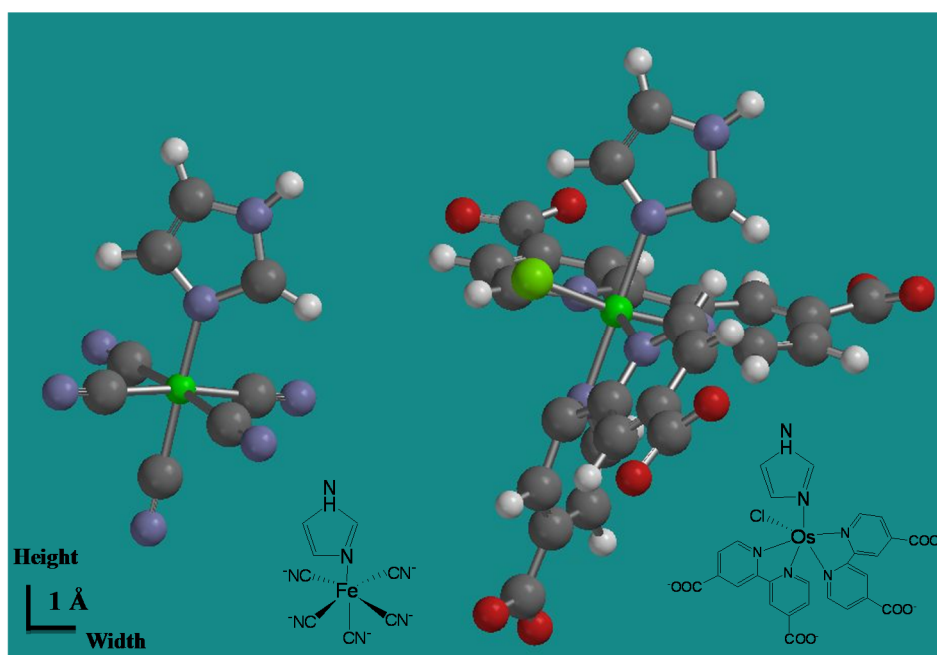


Figure 1-4 Molecule model of imidazole-Fe[(CN)₅] (left) and imidazole-Os[(dcbppy)₂Cl] (right) drawn on Spartan molecular modeling software (Wavefunction, Inc., USA). The sizes of Fe[(CN)₅] and Os[(dcbppy)₂Cl] were estimated as $7.2 \times 6.2 \text{ \AA}^2$ and $11.2 \times 13.8 \text{ \AA}^2$ (height \times width), respectively.

SOD is a kind of flavoenzymes; the global charge of SOD ($pI = 4.9$, Toyobo Co.) at pH 7 is negative but the surface electrostatic potential near the redox center is slightly positive (Fig. 1-5). Therefore, some MET activity against SOD might be expected even for negatively charged metal complex-containing polymers. However, no catalytic current was observed for both PVI[Os(dcbppy)₂Cl] and PVI[Fe(CN)₅]. In addition, the MET activity of PVI[Os(dmebpy)₂Cl] was extremely low compared to that in the GOD reaction (Table 1-1). The poor MET activity of these redox polymers seems to be ascribed to the steric hindrance in the reaction with SOD, since the FAD of SOD is deeply buried in the molecule; moreover, the path of the channel to the FAD with positive surface potential outside comprises hydrophobic residues [34]. The steric effect and electrostatic repulsion with the tunnel are enhanced by the polymerization. Anyway, PVI[Fe(CN)₅] as well as PVI[Os(dcbppy)₂Cl] may be utilized as a mediator of SOD/POD-based bienzyme biosensors.

All the redox polymers used did not give any catalytic current for ChOD. The disappearance of the MET activity by the polymerization seems to be ascribed to the steric hindrance as in the case of SOD. The FAD is buried near the center of the subunit of the homodimer and only 2.1% of the FAD surface area is exposed to the solvent [35], therefore even PVI[Os(dmebpy)₂Cl] is hard to react with the FAD of ChOD.

LOD with flavin mononucleotide (FMN) as the redox center was also examined. The surface electrostatic potential of the tunnel to the FMN of LOD is positive although the entrance is slightly negatively charged (Fig. 1-6). In addition, the FMN located on the bottom of the tunnel is somewhat accessible to solvent compared with the FAD in ChOD and SOD. Therefore, the steric hindrance does not seem to be so strong as SOD and ChOD, and the negatively charged electron acceptor may also react with LOD. Actually, the rate constant of LOD with ferricyanide ($5.7 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ in pH 7.5, 0.1 M PBS at 25 °C) is approximately one order larger than that of GOD ($3.2 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ in pH 7.0, 0.1 M phosphate/citrate at 25 °C) [16, 36].

As expected from the structural information, PVI[Os(dcbppy)₂Cl] gave clear catalytic current in the presence of LOD and lactate (Fig. 1-7A) and even PVI[Fe(CN)₅] gave catalytic current although it was very small (Fig. 1-7B). However, the larger catalytic current was observed with PVI[Os(dmebpy)₂Cl]. The MET activity of these redox polymers is summarized in Table 1-1.

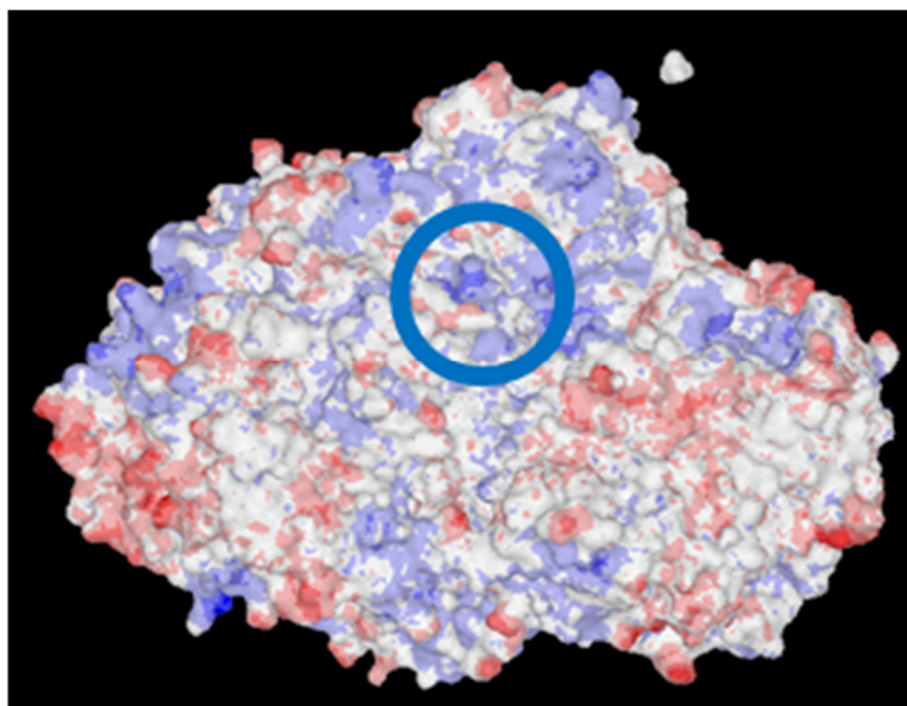


Figure 1-5 Surface electrostatic potential of SOD. The molecule in the blue circle represents FAD. (PDB: 3AD9)

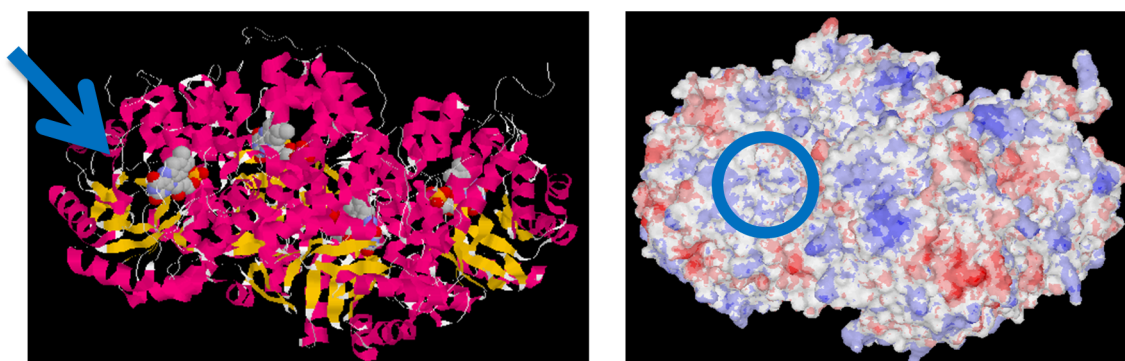


Figure 1-6 Overall structure of LOD (tetramer). Left: ribbon diagram of LOD. Pink ribbon and yellow ribbon represent alpha helix and beta sheet, respectively. The four molecules are the redox center, FMN. Blue arrow indicates the possible entrance for solvent to one of FMN. Right: surface electrostatic potential of LOD. The circle indicates the position of FMN in one of the subunits. (PDB: 2DU2)

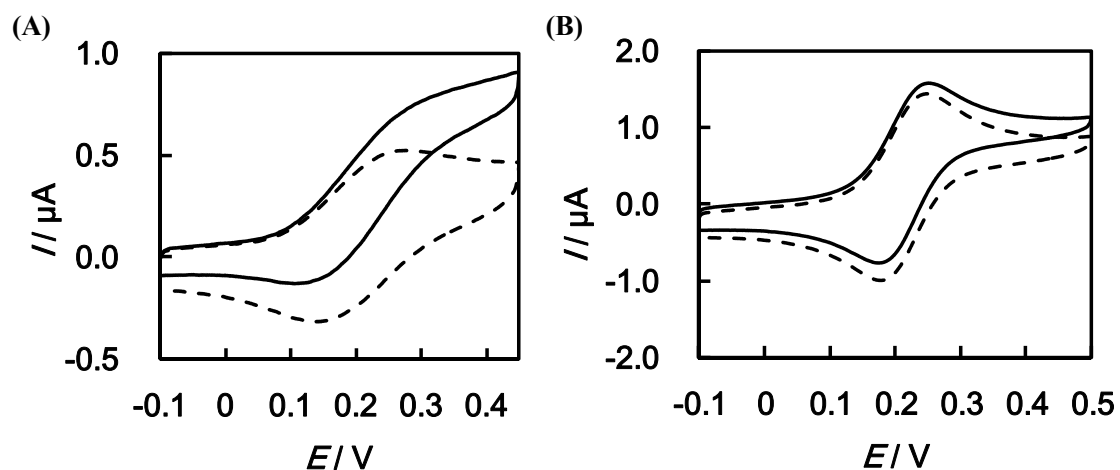


Figure 1-7 Cyclic voltammograms of 11.8 mM lactate solution containing (A) PVI[Os(dcbppy)₂Cl], (B) PVI[Fe(CN)₅] in the absence (dashed line) and presence (solid line) of LOD (40.4 U mL⁻¹) at a scan rate of 20 mV s⁻¹.

Table 1-1 Values of $\log(k_{\text{cat}}/K_{\text{M}})$ for the substrate oxidation catalyzed by flavoenzymes with mediator-containing polymers

	$E^{\circ'}$ (V)	$\log(k_{\text{cat}}/K_{\text{M}})$			
		GOD	SOD	ChOD	LOD
PVI[Os(dmebpy) ₂ Cl]	0.156	7.1	4.3	N/D	6.2
PVI[Os(dcbppy) ₂ Cl]	0.204	6.2	N/D	N/D	5.7
PVI[Fe(CN) ₅]	0.213	N/D	N/D	N/D	4.7

On the other hand, it was found that $\text{PVI}[\text{Fe}(\text{CN})_5]$ works as a good mediator for POD to reduce H_2O_2 as shown in Fig. 1-8. The protoheme, the redox center of POD, locates near the enzyme surface; the surface electrostatic potential near the protoheme is positively charged and the entrance channel into the protoheme for solvent is widely open (Fig. 1-9). Therefore, the high MET activity of $\text{PVI}[\text{Fe}(\text{CN})_5]$ to POD is reasonably understood. Similar situation is observed for $\text{PVI}[\text{Fe}(\text{CN})_5]$ to BOD [18], of which the type 1 redox center locates near the enzyme surface.

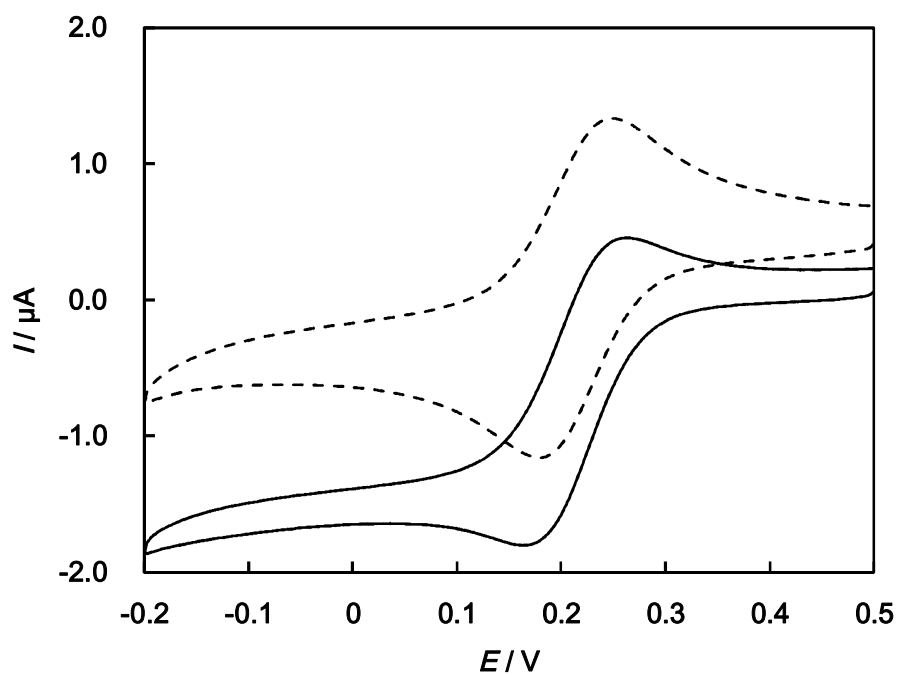


Figure 1-8 Cyclic voltammograms of a solution containing POD (205.6 U mL⁻¹) and PVI[Fe(CN)₅] in the absence (dashed line) and presence (solid line) of 2 mM H₂O₂ at a scan rate of 20 mV s⁻¹.

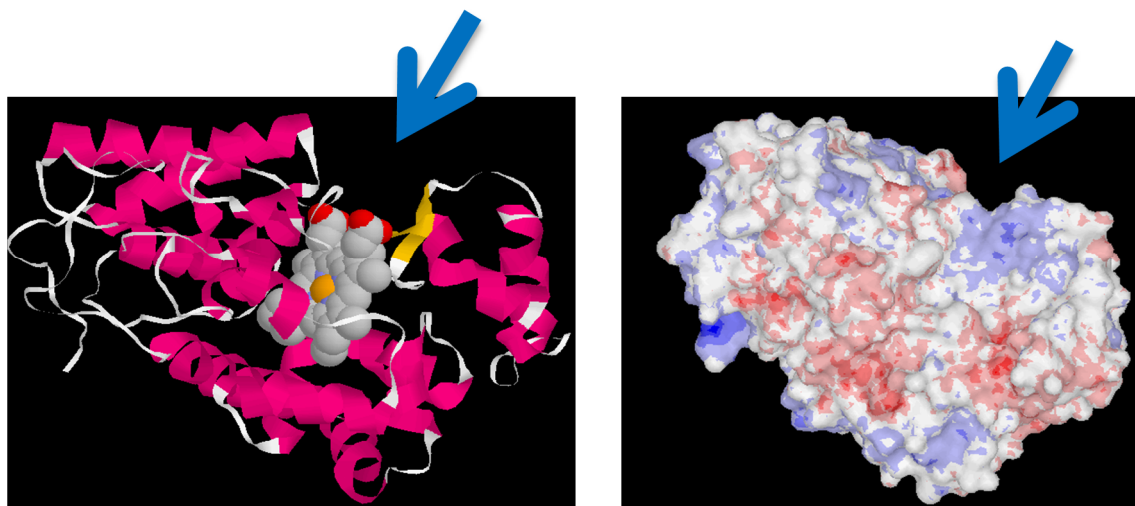


Figure 1-9 Overall structure of POD. Left: ribbon diagram of POD. The space filled molecule is the redox center, protoheme. Right: surface electrostatic potential of POD. Blue arrows indicate the possible entrance for solvent to the protoheme. (PDB: 1W4W)

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Chapter 2 Four enzyme-based biosensor mediated by PVI[Fe(CN)₅]

for creatinine determination

PVI[Fe(CN)₅] (PVI = poly(1-vinylimidazole)) was selected as a mediator for amperometric creatinine determination based on the reductive H₂O₂ detection. Creatinine amidohydrolase (CNH), creatine amidohydrolase (CRH), sarcosine oxidase (SOD), peroxidase (POD), and PVI[Fe(CN)₅] were crosslinked with poly(ethylene glycol) diglycidyl ether (PEGDGE) on a glassy carbon electrode for a creatinine biosensor fabrication. Reduction current was monitored at -0.1 V in the presence of creatinine and O₂. It is revealed that PVI[Fe(CN)₅] is suitable as a mediator for a bioelectrocatalytic reaction of POD, since PVI[Fe(CN)₅] neither reacts with reactants nor works as an electron acceptor of SOD. The amounts of PVI[Fe(CN)₅], PEGDGE, and enzymes were optimized towards creatinine detection. Nafion as a protecting film successfully prevented the enzyme layer from interferences. The detection limit and linear range in creatinine determination were 12 μ M and 12 – 500 μ M ($R^2 = 0.993$), respectively, and the sensitivity was 11 mA cm⁻² M⁻¹, which is applicable for urine creatinine tests. The results of the creatinine determination for four urine samples measured with this proposed method were compared with Jaffe method, and a good correlation was obtained between the results.

Introduction

Creatinine is the final product of creatine metabolism in muscle of mammals and is mainly filtered out of blood in kidneys. The creatinine levels are related to the state of renal function, thyroid malfunction, and muscular disorders. The physiologically normal concentration ranges of creatinine in serum and urine are 40 – 150 μ M and 2.5 – 23 mM, respectively; high creatinine level may result from renal impairment, while the low creatinine level indicates decreased muscle mass [1, 2]. The determination of urine creatinine is also important in other disease measurements since it is widely used as a

calibration index for evaluating disease markers based on the constant excretion rate every day [3]. The current clinical determination of creatinine is based on colorimetric Jaffe reaction, which involves the formation of red products with picric acid in alkaline solution [4]. However, Jaffe method shows poor selectivity since it is affected by numerous metabolites containing carbonyl group found in biological samples, such as glucose, bilirubin, and ascorbic acid (AA) [5, 6]. To increase specificity, creatinine deiminase (CD) has been utilized to generate ammonia for amperometric detection though it is interfered from endogenous ammonia [7, 8].

Rather than CD, creatinine amidohydrolase (CNH), creatine amidohydrolase (CRH), and sarcosine oxidase (SOD) have more widely been utilized for creatinine determination in amperometric method based on the detection of oxygen consumption or generated H_2O_2 [9, 10]. However, as mentioned in *General Introduction*, the signal response is seriously influenced by the concentration of dissolved oxygen and the high operating potential oxidizes other electroactive metabolites.

The second generation biosensors also have been evolved by using mediators to regenerate oxidized SOD [11]. Various kinds of redox mediators such as DCPIP, PMS, ferricyanide, and benzoquinone were utilized for the SOD reaction [12, 13]. Nevertheless, besides the requirement of oxygen removal, the mediating capabilities of DCPIP, PMS and ferricyanide for SOD reaction are not good, and in our knowledge, most of quinones react with sarcosine to generate colored products (see more details in *Appendix*).

On the contrary, mediated biosensors coupled with POD have been mentioned before that they allow the H_2O_2 determination at low operating potentials around 0 V to avoid the interference oxidation with high sensitivity and high stability [14-16]. In this chapter, PVI[Fe(CN)₅] (PVI = poly(1-vinylimidazole)) was selected as a mediator for creatinine determination considering its poor mediating capability against SOD. The principle of the creatinine detection is shown in Scheme 2-1. The three enzymes, POD, and PVI[Fe(CN)₅] were crosslinked with PEGDGE on a glassy carbon (GC) electrode and the reactions are shown in Fig. 2-1. Creatinine was hydrolyzed and oxidized to generate H_2O_2 , then the reduction current of PVI[Fe(CN)₅] was observed at -0.1 V. The catalytic effect of PVI[Fe(CN)₅] on SOD and POD, electrode optimization, interference effect, and the comparison with Jaffe method will be described.

Experimental

Reagents

The sources of some chemicals were mentioned in *Chapter 1*. Creatine, creatinine, and saturated picric acid solution were obtained from Wako Chem. Co. (Osaka, Japan). CRH from microorganism (13 U mg^{-1}) and CNH from microorganism (258 U mg^{-1}) were purchased from Toyobo Co. (Osaka, Japan). Nafion (5 wt% in mixture of lower aliphatic alcohols and water, contains 45% water), acetaminophen, and dopamine were from Sigma-Aldrich (USA). Uric acid (UA) solution was prepared by dissolving in 10 mM NaOH, and the enzymes, substrates, several interferences and PEGDGE solutions were prepared using 100 mM phosphate buffer (pH 7.0). Other chemicals were of analytical grade and used as received. Urine samples were donated from healthy volunteers. The synthesis of PVI[Fe(CN)₅] was described in *Chapter 1*.

Fabrication of enzymes and PVI[Fe(CN)₅]-modified electrode

The surface of a GC electrode (3 mm diameter, BAS) was polished with alumina powder, washed with distilled water and dried before use. Two μL of PVI[Fe(CN)₅], 1 μL of PEGDGE, and 2 μL of enzyme solution were successively cast onto the surface of GC electrode and well mixed with a syringe needle. The electrode was dried at 4 °C for 24 h. Before measurements, the proposed electrode was immersed into 100 mM phosphate buffer (pH 7.0) for at least 30 min. For interference tests, 5 μL of 1% Nafion in ethanol was cast onto the surface of the proposed electrode and air-dried before immersing the electrode into buffer for pre-conditioning.

Electrochemical measurements

All electrochemical investigations were carried out in 100 mM phosphate buffer (pH 7.0) under moderate stirring at 25 °C with an electrochemical analyzer (BAS CV 50 W, BAS Inc., Japan). A platinum wire electrode and an Ag|AgCl|sat. KCl electrode were used as the counter and reference electrodes, respectively.

Creatinine determination by Jaffe method

This electro-enzymatic method was compared with spectrophotometric Jaffe method [17]. One hundred μL of urine sample or creatinine standard solution ($0.5 - 2.5 \text{ mg mL}^{-1}$) prepared in a 10 mM HCl solution was added into a reagent solution containing 2 mL of saturated picric acid solution and 150 μL of 10 wt% NaOH. After 10-min incubation at room temperature, 7.75 mL of distilled water was added into the test solution. After 5.0 min, the absorbance at $\lambda = 520 \text{ nm}$ was measured with a spectrophotometer (MultiSpec-1500, Shimadzu Co., Japan).

Results and Discussion

Catalytic effect of PVI[Fe(CN)₅] on SOD and POD

In oxidase/POD bienzyme system, the mediator oxidized in the POD reaction may also be reduced by receiving the electron from the reduced oxidase generated in the substrate oxidation, which interferes with the detection of mediator reduction on the electrode. To evaluate the mediating effect for mediator selection, PVI[Fe(CN)₅] and PVI[Os(dmebpy)₂Cl] (dmebpy = 4,4'-dimethyl-2,2'-bipyridine) were used to investigate the interactions with SOD and POD. The cyclic voltammetric responses of SOD/POD-PVI[Fe(CN)₅] electrode and SOD-PVI[Fe(CN)₅] electrode are shown in Fig. 2-2. In Fig. 2-2A, PVI[Fe(CN)₅] did not mediate the SOD reaction, while the catalytic reduction current from the POD reaction was clearly observed (Fig. 2-2B). On the other hand, PVI[Os(dmebpy)₂Cl] reacted with SOD; the catalytic oxidation current of creatinine was obtained (Fig. 2-2C). Therefore, in the cyclic voltammogram of SOD/POD-PVI[Os(dmebpy)₂Cl] electrode, the catalytic reduction current from POD reaction was hardly observed as shown in Fig. 2-2D.

The reason which causes the difference in the reactivity between PVI[Fe(CN)₅] and PVI[Os(dmebpy)₂Cl] was explained as in *Chapter 1*. Briefly, the meditating capability of hexacyanoferrate ion on the SOD reaction is originally very low because of the steric hindrance effect and the electrostatic repulsion between negatively charged hexacyanoferrate and the deeply buried FAD of SOD [18]. After binding pentacyanoferrate with PVI, it may become more difficult to enter into the active site of SOD due to the increased charge density and fixation. For this reason, there is no mediating effect of PVI[Fe(CN)₅] on the SOD reaction. On the other hand, Os(dmebpy)₂Cl is more hydrophobic than pentacyanoferrate, which decreases the difficulty in entering the active site of the oxidase. In POD reaction, both of the polymers can transfer electrons to the protoheme, the redox center of POD, because the location of protoheme is near the surface of POD, and the size of POD is smaller than that of SOD, which shortens the distance between the mediator and the redox center [19]. Therefore, it is easier for PVI[Fe(CN)₅] to react with POD than with SOD. Thus,

the data in Fig. 2-2 clearly confirm the concept that PVI[Fe(CN)₅] has extremely low reactivity against SOD, while it has high reactivity against POD; the linear range of H₂O₂ measured by the POD-PVI[Fe(CN)₅] sensor was from 3 to 65 μM and the sensitivity was approximately 410 mA cm⁻² M⁻¹ (data not shown). Based on the results, PVI[Fe(CN)₅] is suitable as a mediator for the SOD/POD bienzyme system, and the signal intensity was practically independent of the oxygen tension at least in the range from 0.2 to 1 atm (data not shown).

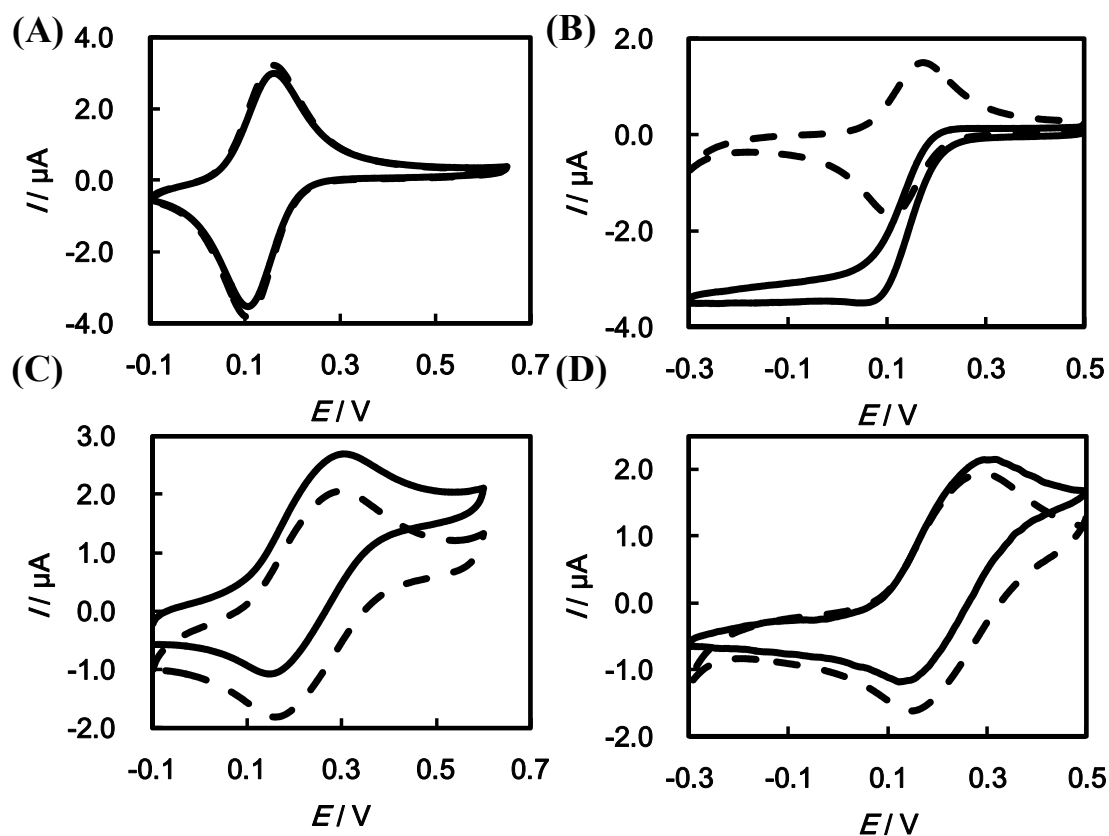


Figure 2-2 Cyclic voltammograms of (A) SOD-PVI[Fe(CN)₅] electrode, (B) SOD/POD-PVI[Fe(CN)₅] electrode, (C) SOD-PVI[Os(dmebpy)₂Cl] electrode, and (D) SOD/POD-PVI[Os(dmebpy)₂Cl] electrode. (A) and (C) were measured in Ar-saturated solutions, while (B) and (D) were measured in air-saturated solutions. The dashed lines represent the measurements in 100 mM phosphate buffer (pH 7.0), while the solid lines represent the measurements in 5 mM sarcosine. Scan rate: 20 mV s⁻¹. Electrode conditions, SOD: 0.83 U, POD: 2.57 U, PEGDGE: 11 μg, PVI[Fe(CN)₅]: 30 μg, PVI[Os(dmebpy)₂Cl]: ca. 40 μg.

Optimization of enzymes and PVI[Fe(CN)₅]-modified electrode

Fig. 2-3 shows the effect of the PVI[Fe(CN)₅] amount fabricated with the four enzymes on the GC electrode. Over 30 µg of PVI[Fe(CN)₅], the amperometric response did not vary dramatically from each other. However, with a large amount of PVI[Fe(CN)₅], the longer time was needed to get the steady state (e.g., 600 s for one injection for the electrode containing 50 µg of PVI[Fe(CN)₅], while 300 s for the electrode containing 30 µg of PVI[Fe(CN)₅]). It indicates that the thick film of the polymer increases the difficulty in the substrate permeation. Considering the current response and the time to reach the steady state, 30 µg of PVI[Fe(CN)₅] was selected in the following experiments.

The weight percentage of PEGDGE was then examined in the range from 2.5% to 38.8% of the total weight of the cast on the electrode. The time to reach the steady state increased with an increase in the percentage of PEGDGE above 11.2%, while the magnitude of the current responses did not change significantly (data not shown). The high percentage of PEGDGE increases the rigidity of the polymer film, resulting in the poor permeability of the substrate. Based on the result, the percentage of PEGDGE was optimized to be 11.2%.

Since the enzyme composition of POD and SOD may affect the biosensing performance, the effect of the POD/SOD ratio on the current response for the creatinine detection was also examined in the range from 0.15 to 1 (w/w). Fig. 2-4 shows that the highest current response was obtained at the ratio of 0.2 (4 µg of POD and 20 µg of SOD) for the detection of 100 µM creatinine, and the current response decreased gradually with an increase in the ratio of POD to SOD. The ratio of POD to SOD was therefore optimized to be 0.2.

The amounts of the four enzymes were then determined as follows: 1.29 U of POD, 0.42 U of SOD, 0.26 U of CRH, and 1.29 U of CNH by considering the effect of the total weight of the enzyme on the current response. The response time was approximately 150 s and the relative error in the reproducibility (in electrode-to-electrode) was 14% with a 90%-confidence level.

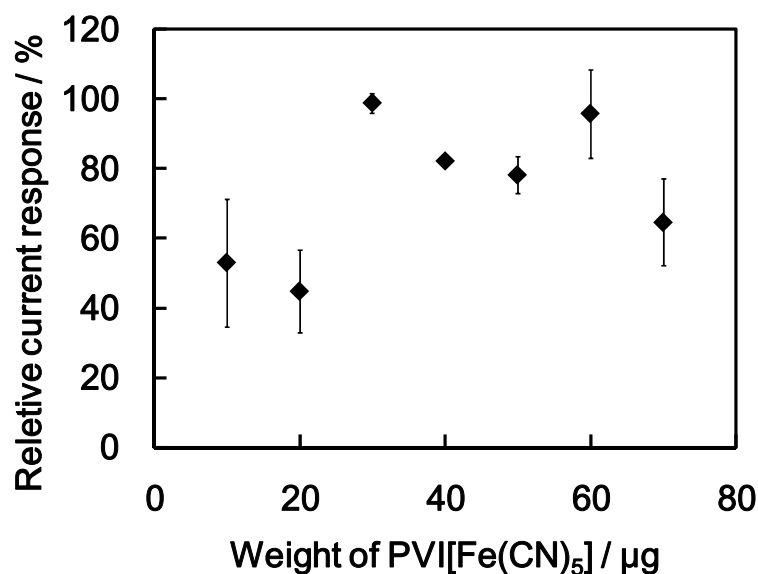


Figure 2-3 Dependence of the current response on the PVI[Fe(CN)₅] amount for the detection of 100 μM creatinine at −0.1 V. Electrode conditions, CNH: 1.29 U, CRH: 0.26 U, SOD: 0.33 U, POD: 1.03 U, PEGDGE: 20 μg. The error bars were evaluated by the Student *t*-distribution with a 90%-confidence level.

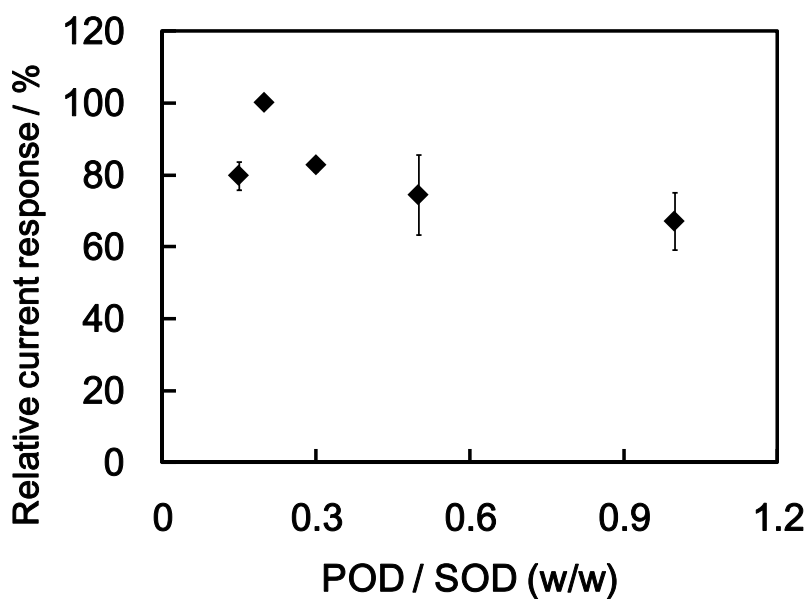


Figure 2-4 Dependence of the amperometric response on the weight ratio of POD to SOD for the detection of 100 μM creatinine at −0.1 V. Electrode conditions, CNH: 1.29 U, CRH: 0.26 U, SOD: 0.33 U (20 μg), PEGDGE: 11.2%, PVI[Fe(CN)₅]: 30 μg. The error bars were evaluated by the Student *t*-distribution with a 90%-confidence level.

Interference effect

The creatinine biosensor based on the reductive H_2O_2 detection at a low operating potential (-0.1 V vs. $\text{Ag}|\text{AgCl}$) minimizes the undesirable oxidation of electroactive interference in physiological fluids.

In order to eliminate the interference effect on POD which is due to the poor substrate specificity mentioned in *General Introduction*, negatively charged Nafion was utilized as a protecting film on the top of the enzymes-PVI[$\text{Fe}(\text{CN})_5$]-modified electrode to exclude anionic species such as AA and UA. The interference effect on the amperometric response measured with the proposed electrode covered with and without Nafion film is shown in Fig. 2-5. For the detection of $150\text{ }\mu\text{M}$ creatinine, the amperometric response with the Nafion-coated electrode was smaller than that with the electrode without Nafion film because of the inhibition of the mass transfer. However, the interference effect was eliminated by the protection of Nafion film, while the current responses of $150\text{ }\mu\text{M}$ UA and $10\text{ }\mu\text{M}$ AA were observed at the electrode without Nafion film. In the case of urine, the normal concentrations of creatinine and UA are in the same level [20], and the concentration of creatinine is about thirty times higher than that of AA [21]. This means that Nafion used as a protecting film satisfies the creatinine determination in real urine samples. Acetaminophen and dopamine were also tested for the interference test. Small interference effects were observed in the solutions containing $150\text{ }\mu\text{M}$ creatinine and $10\text{ }\mu\text{M}$ acetaminophen (recovery = $108 \pm 7\%$) or dopamine (recovery = $96 \pm 10\%$). Considering the fact that the concentrations of these interferences are much smaller than that of creatinine, these interferences do not seriously affect the creatinine detection in this biosensing system.

Internal creatine in urine might also interfere with creatinine determination since it reacts with CRH immobilized on the enzymes-PVI[$\text{Fe}(\text{CN})_5$]-modified electrode to result in the overestimation of creatinine. In the case of urine, the excretion rate of creatinine is about twenty times higher than that of creatine [22]. In our experiment, as the concentration ratio of creatine to creatinine is 6.7% ($10\text{ }\mu\text{M}$ creatine/ $150\text{ }\mu\text{M}$ creatinine), the signal ratio of creatine to creatinine is only about 2% (data not shown) though H_2O_2 generation from creatine requires fewer enzymatic steps than that from creatinine. It may be described that in neutral pH, creatinine is positively charged while

creatine is a zwitterion, therefore creatinine is easier to penetrate the negatively charged Nafion film into the enzyme layer to get a larger amperometric response [23]. As a result, the internal creatine in urine does not significantly affect the creatinine determination in our method.

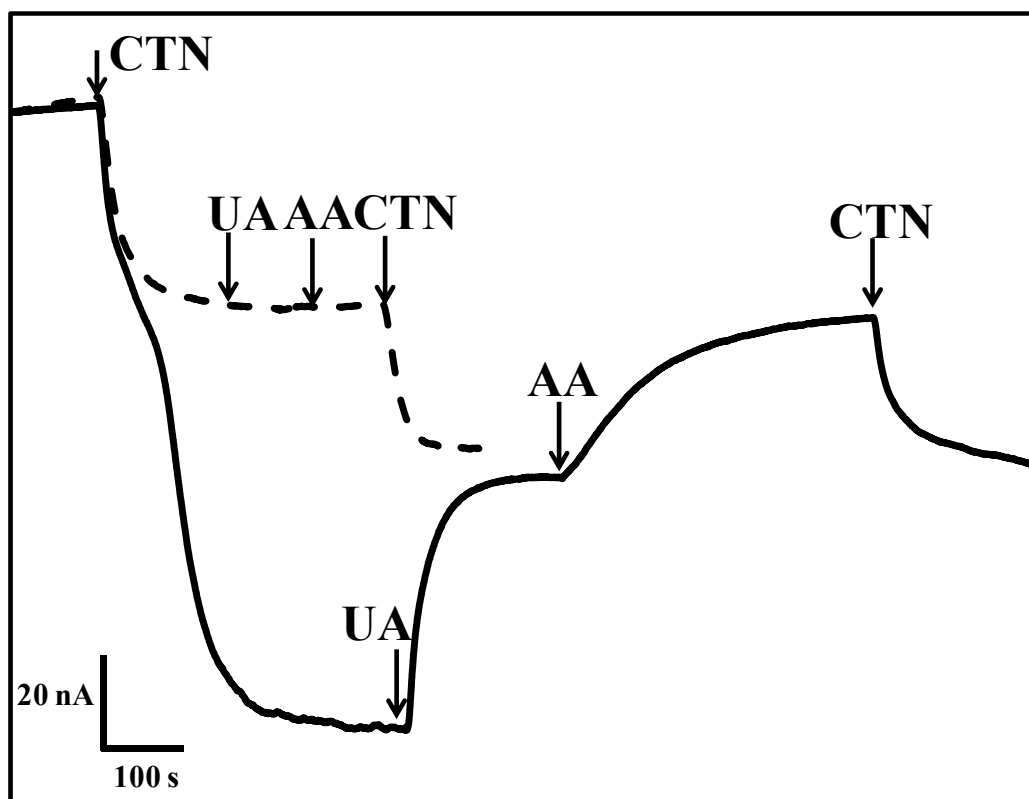


Figure 2-5 Amperometric responses of the proposed electrodes without Nafion (solid line) and with 5 μL of 1% Nafion (dashed line). CTN: 150 μM creatinine, UA: 150 μM , AA: 10 μM . The arrows indicate the injection time of the respective solutions. Electrode conditions, CNH: 1.29 U, CRH: 0.26 U, SOD: 0.42 U, POD: 1.29 U, PEGDGE: 11.2%, PVI[Fe(CN)₅]: 30 μg . Operating potential: -0.1 V .

Comparison with Jaffe method

The amperometric response for the optimized biosensing electrode at -0.1 V is presented in Fig. 2-6. The detection limit of creatinine is $12\text{ }\mu\text{M}$ ($S/N > 3$) and the linear range is from 12 to $500\text{ }\mu\text{M}$ ($R^2 = 0.993$), and the sensitivity is $11\text{ mA cm}^{-2}\text{ M}^{-1}$, which is sufficient for urine sample test. This method was applied to the creatinine determination of urine from four volunteers and was compared with Jaffe method which is widely used in clinical diagnosis. In the electro-enzymatic method, $10\text{ }\mu\text{L}$ of urine sample was injected into 1 mL of 100 mM phosphate buffer ($\text{pH } 7.0$) for measurements. Table 2-1 shows the creatinine concentrations evaluated from the absorbance at $\lambda = 520\text{ nm}$ based on Jaffe method and from the current response at -0.1 V measured by this proposed method, respectively. Numbers 1 and 2 are the urine samples which were donated by male, while No. 3 and 4 were donated by female. The data show that the concentration of urine creatinine in male is higher than that in female, which is consistent with the typical human reference ranges, and a good correlation is obtained between the two methods. The values measured by the electro-enzymatic method are lower than Jaffe method, most probably because other compounds in urine (UA or AA) caused positive interference in Jaffe method. The results also show that the creatinine concentrations of No. 1 and 2 measured by Jaffe method are similar with each other, while the value of No. 2 is smaller than that of No. 1 measured by this method. This seems to be resulted from a high concentration of interference in No. 2, which reacted with picric acid to overestimate the creatinine concentration in Jaffe method.

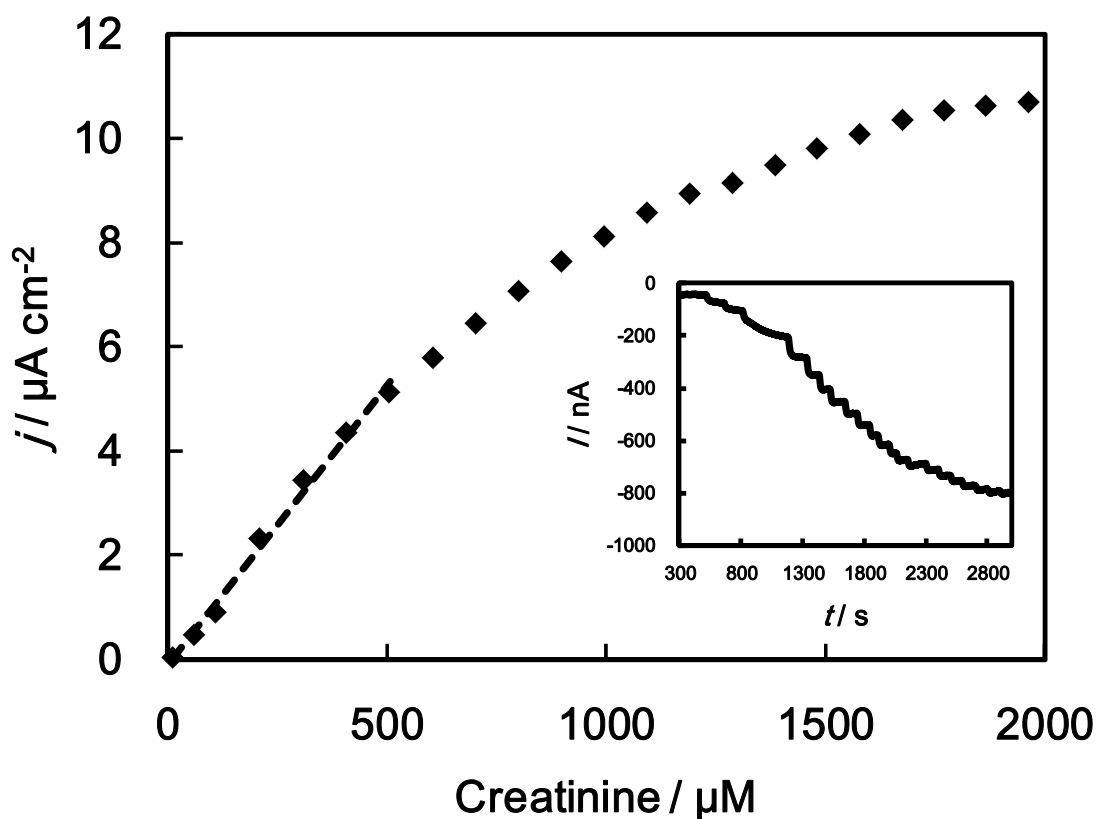


Figure 2-6 Dependence of the amperometric response on the creatinine concentration at -0.1 V. Electrode conditions were the same as those in Fig. 2-5 with Nafion film. The broken line is a linear regression curve; current density ($\mu\text{A cm}^{-2}$) = $11 (\text{mA cm}^{-2} \text{M}^{-1}) \times [\text{creatinine}] (\mu\text{M}) - 120 (\text{nA cm}^{-2})$, $R^2=0.993$. The inset shows the amperometric response.

Table 2-1 Determination of creatinine from urine samples

Number	Creatinine / mM	
	Jaffe method	This method
1	17.0 ± 0.5	13.3 ± 0.8
2	17.1 ± 0.5	11.9 ± 0.2
3	5.7 ± 0.2	5.2 ± 0.3
4	11.6 ± 0.5	9.1 ± 0.7

The errors were evaluated by the Student t -distribution with a 90%-confidence level.

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Chapter 3 Sensitive D-amino acid bienzyme biosensor mediated by

PVI[Fe(CN)₅]

A sensitive D-amino acid oxidase (DAAO)/peroxidase (POD) bienzyme biosensor is constructed, in which PVI[Fe(CN)₅] (PVI = poly(1-vinylimidazole)) is selected as a mediator. Reduction current of PVI[Fe(CN)₅] related to the H₂O₂ concentration generated in the DAAO reaction was measured at -0.1 V vs. Ag|AgCl with a DAAO/POD-PVI[Fe(CN)₅] electrode. The result revealed that PVI[Fe(CN)₅] is suitable as a mediator for this bienzyme system because of its appropriate formal potential and its extremely low reactivity against DAAO. The stability of DAAO was improved by adding free flavin adenine dinucleotide and the electrode composition was optimized for the detection of D-alanine. Nafion and ascorbate oxidase-immobilized films worked successfully to prevent severe interference from uric acid and ascorbic acid. The low detection limits of D-alanine (2 μM) and D-serine (2 μM) imply its possibility for the determination of extremely low concentration of D-amino acids in physiological fluids. The proposed bienzyme biosensor is proved to be capable of detecting D-amino acids in urine.

Introduction

The role of D-amino acids (DAAs) has become of great importance in life science, since several DAAs are found to have physiological functions in mammals. With regard to the central nervous system in brain, D-serine acts as a co-agonist of *N*-methyl-D-aspartate receptor, which is associated with learning and memory [1], and D-alanine in the anterior pituitary gland, pancreas, and plasma might have a physiological function to the insulin regulation [2]. The concentration of DAAs also shows a strong correlation to some diseases. For example, the ratio of D-serine to the total (D- + L-) serine concentration in the serum of Alzheimer's disease patients was reported to be lower than that of normal subjects [3]. As the dynamic monitoring of the DAAs concentration

is of acute interest, a rapid, simple and highly sensitive method for the DAA detection is essential for further study. Separation-based analytical methods such as high performance liquid chromatography (HPLC) and capillary electrophoresis are conventionally used for DAA determination with high sensitivity [4, 5]. D-Amino acid oxidase (DAAO) oxidizes DAAs to the corresponding imino acids, which have been further utilized to react with *o*-phenylenediamine and 2-mercaptoethanol to form the corresponding fluorescent quinoxalinol derivatives. The fluorescent derivatives can be separated by HPLC, and this method eliminates the interference from extremely high concentrations of L-amino acids [6, 7]. Considering the convenience for *in vivo* detections and the undesirability of cumbersome instruments, enzyme-based amperometric biosensors are favored for DAA detection. DAAO is a flavoenzyme and is widely used in enzymatic biosensors for DAA determination, either based on the direct detection of generated H_2O_2 [8-10] or the detection of artificial mediators used as electron acceptors of the substrate-reduced DAAO [11, 12]. However, as mentioned in *General Introduction*, the oxidation of both H_2O_2 and mediators requires high operation potentials, which always accompanies the co-oxidation of other electroactive metabolites in physiological fluids. In addition, the exclusion of O_2 in the case of mediated biosensors seems to be impractical for *in vivo* analysis.

On the other hand, peroxidase (POD) in combination with H_2O_2 -producing oxidases including DAAO has been studied intensively in recent years for the substrate detection in the way of direct or mediated electron transfer [13, 14]. Since the low potential operation decreases the background current and noise levels, and eliminates the undesirable oxidation of electroactive interferences, bienzyme biosensors show high sensitivity and stability. Nevertheless, the oxidized mediators may also act as electron acceptors of H_2O_2 -producing oxidases based on the dehydrogenase activity of the oxidase just mentioned in *General Introduction*.

Here, the author focuses the attention on $\text{PVI}[\text{Fe}(\text{CN})_5]$ (PVI = poly(1-vinylimidazole)) as a mediator for the DAAO/POD bienzyme biosensor considering its suitable operating potential and the poor mediating capability for other flavoenzymes. The interactions of $\text{PVI}[\text{Fe}(\text{CN})_5]$ with POD and DAAO will be studied, and the electrode optimization and the interference effect will also be investigated.

Experimental

Reagents

The sources of some chemicals were mentioned in *Chapter 1* and *2*. D-Serine, glutaraldehyde (GA, 20%), and flavin adenine dinucleotide (FAD) disodium salt were obtained from Wako Chem. Co. (Osaka, Japan). DAAO from porcine kidney (8.2 U mg^{-1}) and D-alanine were from Sigma-Aldrich (USA). Ascorbate oxidase from *cucumis* sp. (AO, 333 U mg^{-1}) was purchased from Toyobo Co. (Osaka, Japan). Uric acid (UA) solution was prepared with 10 mM NaOH; FAD was dissolved in distilled water, and the enzymes, substrates, ascorbic acid (AA) and poly(ethylene glycol) diglycidyl ether (PEGDGE) solutions were prepared with 30 mM potassium phosphate buffer (pH 7.0). Other chemicals were of analytical grade and used as received. The urine sample was donated from a healthy volunteer.

The synthesis of $\text{PVI}[\text{Fe}(\text{CN})_5]$ was described in *Chapter 1* except that the stock solution of $\text{PVI}[\text{Fe}(\text{CN})_5]$ was prepared in distilled water.

Co-immobilization of POD, DAAO and $\text{PVI}[\text{Fe}(\text{CN})_5]$

A solution containing $\text{PVI}[\text{Fe}(\text{CN})_5]$, PEGDGE, POD and DAAO was cast onto the surface of a glassy carbon electrode (3 mm diameter, BAS) and well mixed with a syringe needle. The electrode was dried at 4 °C for 24 h. Before measurements, the proposed electrode was immersed into a 30 mM potassium phosphate buffer (pH 7.0) for 20 min.

Fabrication of interference-free bienzyme electrode

To improve the stability of DAAO, 1 μL of 400 μM FAD solution (4×10^{-10} mol, ca. 0.3 μg) was cast onto the aforementioned electrode (see *Results and Discussion* for details). For interference tests, 3 μL of Nafion solution diluted by distilled water was then added onto the electrode after air-drying. Unbounded polymer and enzymes were removed by dipping the electrode into distilled water and 3 μL of diluted Nafion solution

was cast onto the electrode again after air-drying. Finally, AO was crosslinked with GA on the electrode at 4 °C for 2 h.

Electrochemical measurements

Electrochemical experiments were carried out in a potassium phosphate buffer (pH 8.0, 30 mM) under stirring at 37 °C with an electrochemical analyzer (611B or 1000, CH Instrument, USA). The reference and counter electrodes were an Ag|AgCl|sat. KCl and a Pt wire, respectively. Cyclic voltammetry was performed at a scan rate of 20 mV s⁻¹.

Oxygen depletion due to the DAAO reaction was measured at 37 °C and -0.6 V with a Clark-type oxygen electrode (Optosience, Japan). The pH 8.0, 30 mM potassium phosphate buffer solution containing 3 µg mL⁻¹ DAAO and 4 mM D-alanine was used for experiments.

Results and Discussion

Reactivities of PVI[Fe(CN)₅] against DAAO and POD

DAAO/POD bienzyme systems may suffer from interference because of the dehydrogenase activity of DAAO. In this sensing system, PVI[Fe(CN)₅] was selected as a mediator. Fig. 3-1A shows that PVI[Fe(CN)₅] has extremely low reactivity against DAAO, while in the POD reaction the catalytic current is clearly observed with PVI[Fe(CN)₅] (Fig. 3-1B). The result indicates the poor interaction between DAAO and PVI[Fe(CN)₅]. The quite different reactivities of PVI[Fe(CN)₅] against DAAO and POD are interpreted as follows: The redox center (FAD) of DAAO locates in hydrophobic surroundings and is deeply buried in the interior to induce the steric hindrance [15]; PVI[Fe(CN)₅] with negatively charged ligands would be difficult to enter into the FAD-catalytic center. Therefore, no obvious mediating effect of PVI[Fe(CN)₅] was observed for the DAAO reaction. On the other hand, as mentioned in *Chapter 1*, the redox center of POD locates near the enzyme exterior with widely open entrance; furthermore, the electrostatic potential around the redox center is positively charged, which makes it easier for PVI[Fe(CN)₅] to shuttle electrons between POD and electrode. Thus, the author can conclude that PVI[Fe(CN)₅] is very suitable as the mediator for the DAAO/POD bienzyme biosensor with high specificity.

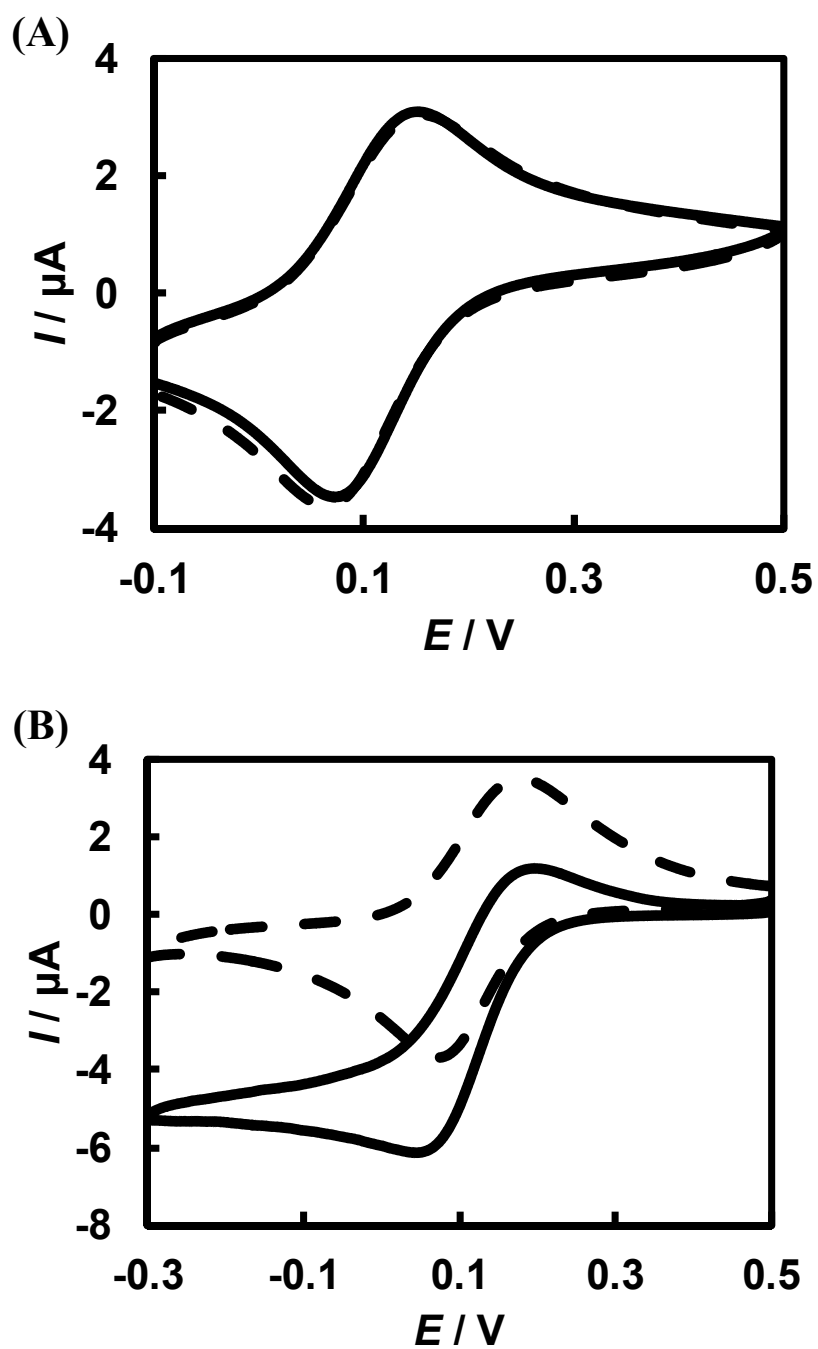


Figure 3-1 Cyclic voltammograms of (A) DAAO-PVI[Fe(CN)₅] electrode in an Ar-saturated solution and (B) DAAO/POD-PVI[Fe(CN)₅] electrode in an air-saturated solution. The dashed lines are the detections in a buffer solution and the solid lines are the detections in 4 mM D-alanine (pH 8.0). Electrode composition, DAAO: 0.33 U, POD: 2.14 U, PEGDGE: 10 μg , PVI[Fe(CN)₅]: 20 μg . Scan rate is 20 mV s^{-1} .

Stability of DAAO/POD-PVI[Fe(CN)₅] electrode

DAAO/POD-PVI[Fe(CN)₅] electrode has been successfully constructed herein for the DAA detection. However, as shown in Fig. 3-2A, the current response of D-alanine decreased with successive operations (open square); the current value in the third detection is only two third of that in the first detection. It was considered that the poor repeatability of the detection is mainly due to the non-covalent binding property of FAD with DAAO [11]. Since the binding between FAD and apo-DAAO is weak, FAD gradually releases from holo-DAAO to the bulk solution to lead the decrease in the DAAO activity. Considering keeping the activity of DAAO, sufficient amount of FAD was added into the experimental solutions to avoid the leakage of FAD from holo-DAAO. The effect of the FAD addition was checked by detecting the oxygen depletion in the DAAO reaction measured with the Clark-type oxygen electrode (Fig. 3-2B). At a low concentration of DAAO without FAD addition (dashed line), the activity of DAAO gradually decreased since FAD was easily released from holo-DAAO to the bulk solution. On the other hand, the oxygen depletion rate of the DAAO reaction increased and DAAO became more stable in the presence of 40 μ M FAD (solid line). The high oxygen depletion rate in the FAD-containing solution indicates that the addition of FAD keeps the DAAO activity. Good repeatability of the proposed biosensing system was then proven by addition of FAD in the test solution, as evidenced in Fig. 3-2A (closed rhombus); no drastic decrease in the current response was observed in the successive experiments. Therefore, the experimental solution containing 40 μ M FAD was selected in the following experiments for other optimization.

Considering the fact that the biosensor sensitivity is related to the deposited polymer thickness, the composition of POD and DAAO, and the total enzyme capacity, the dependence of the current response on the amount of PVI[Fe(CN)₅], the ratio of POD to DAAO, and the weight ratio of the enzymes to the total weight were examined. Fig. 3-3A shows the effect of the PVI[Fe(CN)₅] amount fabricated with 0.16 U DAAO and 2.1 U POD. The amperometric response did not vary drastically from each other over 20 μ g of PVI[Fe(CN)₅], therefore, 20 μ g of PVI[Fe(CN)₅] was selected in the following experiments. The effect of the ratio of POD to DAAO is shown in Fig. 3-3B with fixing the weight of DAAO at 20 μ g (0.16 U). The amperometric response of 5 μ M

D-alanine reached approximately 60 nA when the ratio was 0.1 and no significant variation was observed over the ratio 0.1. Fig. 3-3C indicated that the amount of the enzyme is sufficient for the reaction when the weight ratio of the enzymes to the total weight reached to 0.6.

The optimized values were then determined as follows: 20 μg of PVI[Fe(CN)₅], 0.1 (the ratio of POD to DAAO), and 0.6 (the weight ratio of the enzymes to the total weight). The final composition of proposed electrode contains 20 μg of PVI[Fe(CN)₅], 10 μg of PEGDGE, 1.07 U of POD and 0.33 U of DAAO.

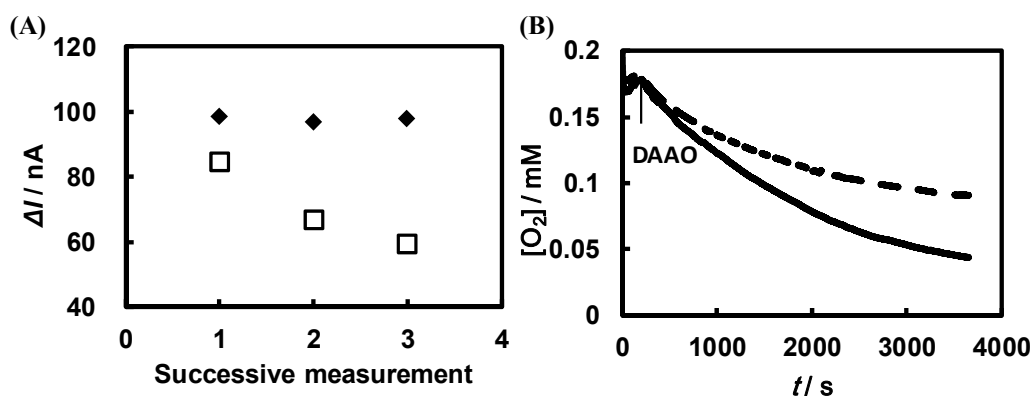


Figure 3-2 (A) Dependence of the current response at -0.1 V on the successive measurements in the solution containing 40 μ M FAD and 5 μ M D-alanine (closed rhombus) or 5 μ M D-alanine only (open square). Electrode composition, DAAO: 0.25 U, POD: 2.67 U, PEGDGE: 10 μ g, PVI[Fe(CN)₅]: 30 μ g. (B) Time dependence of the dissolved oxygen concentration during the DAAO reaction in the solution containing 40 μ M FAD and 4 mM D-alanine (solid line) or 4 mM D-alanine only (dashed line).

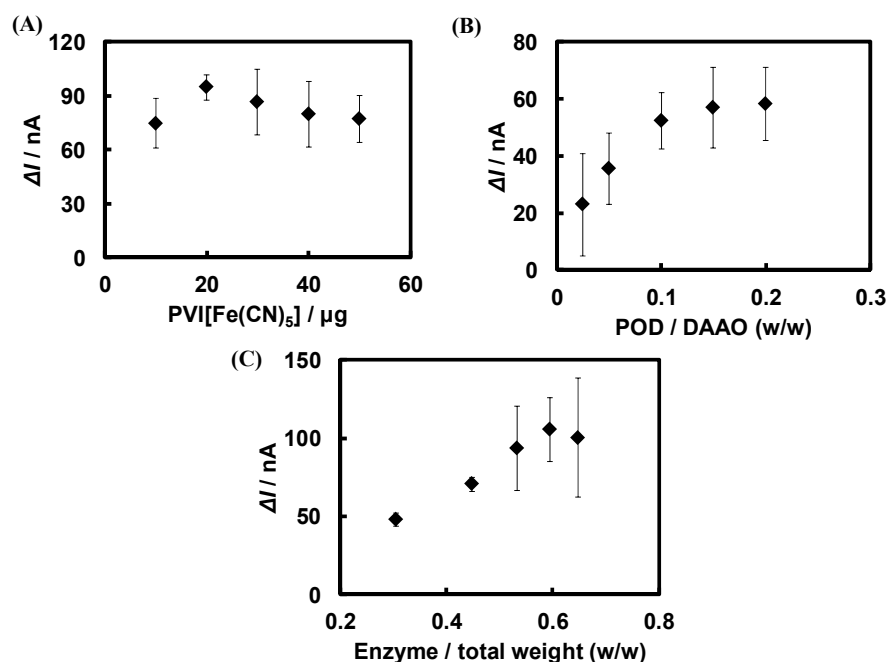


Figure 3-3 Dependence of the amperometric response on the (A) amount of PVI[Fe(CN)₅], (B) the weight ratio of POD to DAAO and (C) the weight ratio of the enzymes to the total weight in the detection of 5 μ M D-alanine at -0.1 V. All experimental solutions contain 40 μ M FAD. The error bars were evaluated by the Student *t*-distribution with a 90%-confidence level.

Interference effect

The DAA bienzyme biosensor at low operating potentials minimizes the undesirable oxidation of electroactive interferences in physiological samples. Considering the poor specificity of POD mentioned in *General Introduction*, negatively charged Nafion was used as a protecting film on the top of the bienzyme electrode to exclude anionic interferences such as AA and UA based on electrostatic repulsion. In addition, 0.3 μg of FAD was cast between the bienzyme layer and the Nafion film in place of the FAD addition in the experimental bulk solution. Furthermore, regarding the low oxidation potential of AA, 15 μg of AO was immobilized with GA on the top of the Nafion film to minimize the interference effect of AA for the purpose of getting high sensitivity. In separated experiments, it has been proved that $\text{PVI}[\text{Fe}(\text{CN})_5]$ works neither as a substrate (reduced form) for AO nor as an electron acceptor of AO in the catalytic oxidation of AA (data not shown). Since $\text{PVI}[\text{Fe}(\text{CN})_5]$ shows little reactivity against AO, AO utilization does not affect the DAA detection. The interference effect on the amperometric response with different concentrations of Nafion is shown in Table 3-1; the current response of 10 μM D-alanine decreased with an increase of the Nafion amount because of the thick film which decreases the mass transfer of the substrate. Comparing the response of D-alanine with that in the AA-containing solution, the relative response increased with an increase of the Nafion amount; thick Nafion film shows a better protecting effect. The relative responses over than 100% with very thick Nafion films may be due to the partial desorption of the Nafion film in succeeding measurements for the DAA+AA mixture, which increases the penetration of D-alanine. Taking the current response and the protecting effect into account, 0.8% Nafion was selected for the biosensor fabrication, and no significant interference was observed in the 10 μM D-alanine solution containing 500 μM UA and 100 μM AA (data not shown). The physiologically normal concentration of UA in urine is about thirty times higher than DAAs, and the normal concentration of AA is in the same level with DAAs [16-18]. Therefore, this bienzyme biosensor protected by Nafion and AO seems to satisfy the DAA determination in urine.

Table 3-1 Effect of Nafion concentration on current response of D-alanine in the solution with or without AA^d

	Nafion (w/v) (%)				
	0.3	0.5	0.8	1.2	1.5
ΔI (D-ala ^a) / nA ^e	152 ± 13	115 ± 3	115 ± 5	61 ± 7	58 ± 10
ΔI (D-ala+AA ^b) / nA ^e	109 ± 15	112 ± 2	112 ± 3	66 ± 3	64 ± 10
Relative response ^c	72%	97%	97%	108%	111%

^a 10 μ M D-alanine.

^b 10 μ M D-alanine + 100 μ M AA.

^c Ratio of ΔI (D-ala + AA) to ΔI (D-ala).

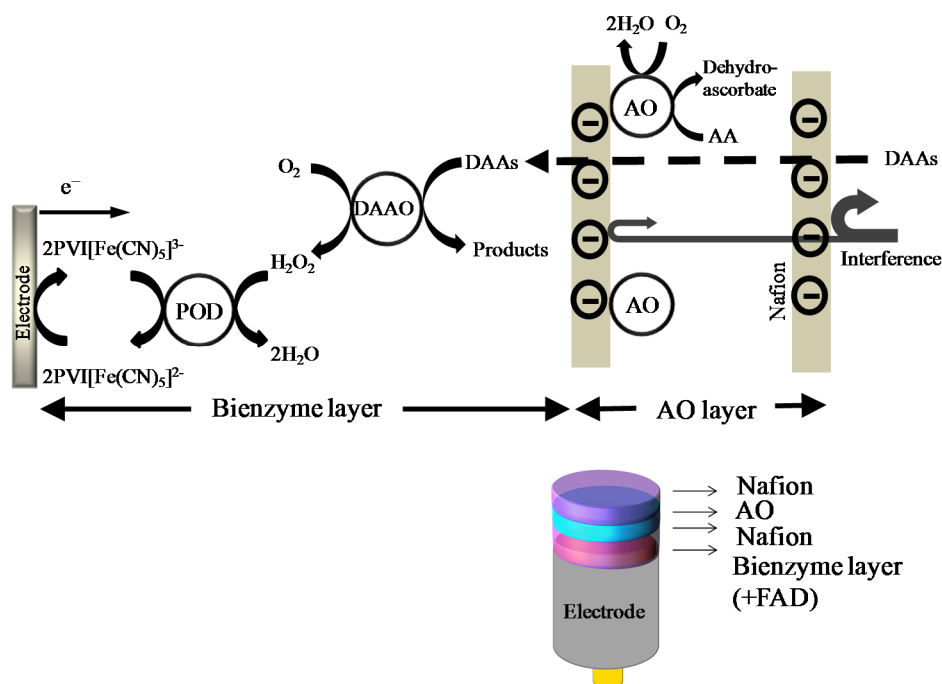
^d Nafion was cast twice on the proposed electrodes. 15 μ g AO was immobilized with 1 μ L of 0.1% GA on all Nafion-covered electrodes.

^e The errors were evaluated by the Student *t*-distribution with a 90%-confidence level.

Sensors for DAAs

The proposed bienzyme biosensor protected by Nafion and AO has successfully worked for the DAA determination in the presence of high concentrations of interference. However, in the preliminary test of urine sample, the sensitivity decreased gradually with successive measurements (data not shown). Some molecules in the urine sample may nonspecifically adsorb on the AO layer of the electrode, which fouled the electrode to affect the sensitivity. To eliminate the nonspecific adsorption, 6 μL of 0.8% Nafion was cast on the top of the AO layer for protection. The illustrated structure of the DAA biosensor is shown in Scheme 3-1. Most of the interferences are inhibited from entering into the sensing part by the outer Nafion film, and the residual UA and AA are blocked by the inner Nafion film and oxidized by AO, respectively. Dopamine and acetaminophen were also tested and only small interference effects were observed in the solutions containing 10 μM D-alanine and 2 μM each of interferences. Considering the fact that the concentrations of these interferences are sufficiently smaller than that of DAA, these interferences would not practically affect the detection in the proposed method. Thus, DAAs can successfully be detected without practical interference effect.

Amperometric responses of two major DAAs in human body, D-serine and D-alanine, are presented in Fig. 3-4, in which the current was measured with the optimized biosensing electrode at -0.1 V . The detection limits of D-serine and D-alanine are both 2 μM ($S/N > 3$); the linear ranges are from 10 to 350 μM and 5 to 150 μM , and the slopes are 5.4 and 9.7 $\text{nA } \mu\text{M}^{-1}$, respectively. It shows that the biosensor has very low detection limit and high sensitivity, which is suitable for the detection of extremely low concentrations of DAAs in physiological samples. The different reactivities of different DAAs indicate that it is difficult for DAAO-based biosensors to determine the total concentration of DAA. Nevertheless, it is still benefit to easily determine the total performance of DAAs with selecting one of DAAs, such as D-alanine, as a standard.



Scheme 3-1 The composition and the reaction sequence of the proposed DAAO/POD bienzyme biosensor protected by Nafion and AO.

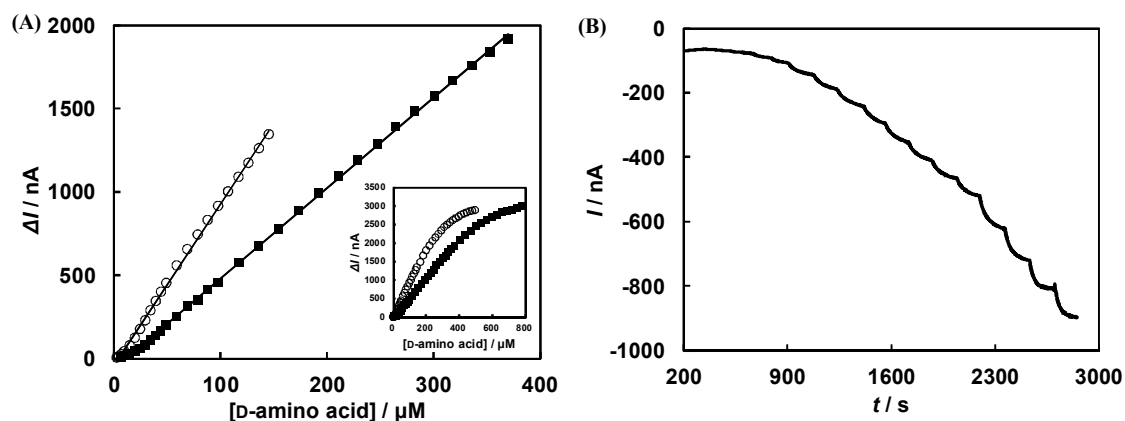


Figure 3-4 (A) Dependence of the amperometric response on the concentration of D-alanine (open circle) and D-serine (closed square) at -0.1V . Current response (nA) = $9.7 (\text{nA } \mu\text{M}^{-1}) \times [\text{D-alanine}] (\mu\text{M}) - 40.7 (\text{nA})$, $R^2=0.999$, and current response (nA) = $5.4 (\text{nA } \mu\text{M}^{-1}) \times [\text{D-serine}] (\mu\text{M}) - 62.3 (\text{nA})$, $R^2=0.999$, respectively. The inset shows the current response in the wide range of the DAA concentration. (B) One part of typical current-time recordings for successive additions of D-serine (with different concentrations).

The standard addition method was then used for a urine sample test. D-Alanine was successively injected into the urine solution which was 100 times diluted with 30 mM potassium phosphate buffer (pH 8.0) and the slope is similar to that measured in the standard D-alanine solution (Fig. 3-5). The concentration of DAAs in the urine was evaluated to be $150 \pm 20 \mu\text{M}$, when D-alanine was selected as a standard, where the error was evaluated by the Student *t*-distribution with a 90%-confidence level. The sensitivity of biosensor is not affected by the interferences in urine.

The proposed method was compared with a method of oxygen consumption measured with a Clark oxygen electrode. The experimental condition of the method of oxygen consumption was described briefly as follows: 150 μL of urine was injected into the 850 μL of buffer solution (pH 8) containing 200 μg DAAO (1.64 U), and the rate of current difference within 50 s was measured at -0.6 V and 37°C . The concentration was evaluated as $120 \pm 50 \mu\text{M}$, the error being evaluated by the Student *t*-distribution with a 90%-confidence level. The difference between the two estimated values may result from the different responses to the different types of DAA in the immobilized enzyme and the free enzyme in the solution. Besides, considering the low concentration of DAA in urine, a large amount of sample (~ 7 times diluted) is needed for the detection of the oxygen consumption, while only 100 times diluted sample is sufficient for the proposed method. Therefore, the other substances in urine may affect the free DAAO's activity in the solution and inhibit the reaction.

The proposed electrode worked properly for at least 24 times experiments (for approximately 1.5 h) without change of the sensitivity. Therefore, it can be concluded that the possible leakage of FAD and AO from the modified electrode is minimized. Considering the application of disposable-type sensing trip, the proposed method may well be utilized in the rather short-term usage. As a result, the DAAO/POD bienzyme biosensor is applicable for clinical diagnosis.

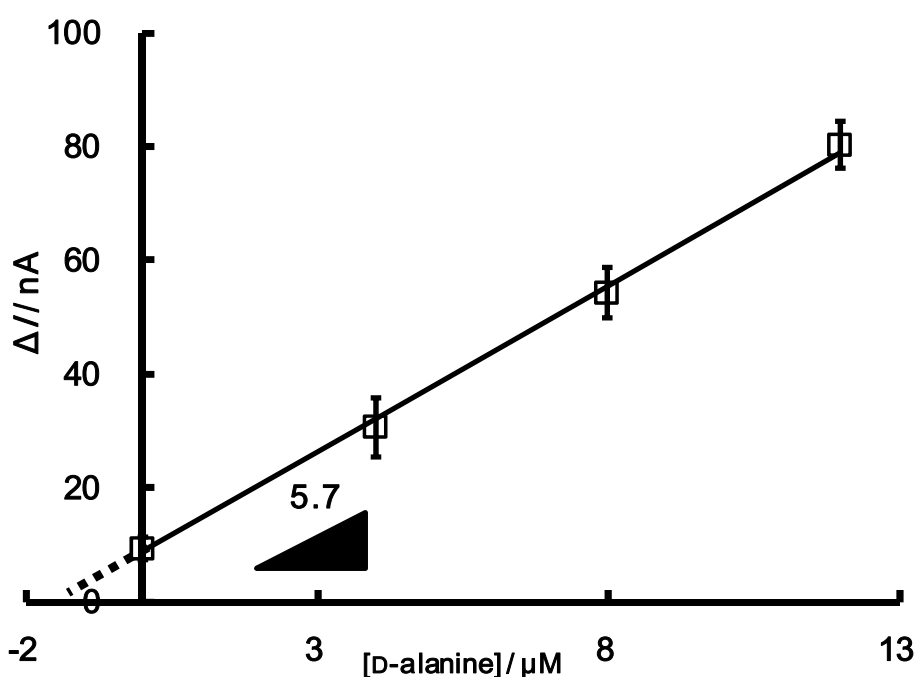


Figure 3-5 Standard addition measurements of a urine sample, which was 100 times diluted with 30 mM potassium phosphate buffer (pH 8.0). Current response (nA) = $5.8 \pm 0.2 \text{ (nA } \mu\text{M}^{-1}) \times [\text{D-alanine}] \text{ (}\mu\text{M)} + 8.8 \pm 1.2 \text{ (nA)}$, $R^2=0.998$. The average values were obtained from triplicate measurements, and the error bars were evaluated by the Student *t*-distribution with a 90%-confidence level. The slope of $5.7 \text{ nA } \mu\text{M}^{-1}$ means the sensitivity of the bienzyme biosensor for the D-alanine detection in the solution without urine.

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Conclusions

Mediated electron transfer (MET)-based biosensors have been developed and utilized for various kinds of clinical diagnosis, and the interference effect due to the oxidation of electroactive compounds in samples is always the problem to be conquered even for electrodes covered with a protecting membrane. Though recently the H_2O_2 -producing oxidase/peroxidase (POD) bienzyme biosensors mediated by redox polymers based on the reductive H_2O_2 detection have been proposed to eliminate the interference oxidation, the cross reaction of mediator with H_2O_2 -producing oxidase drastically interferes with the detection to cause the underestimation. Therefore, in bienzyme biosensors, it becomes of great importance to find a suitable mediator which has the high specificity for POD reaction only.

To find the suitable mediator, in *Chapter 1*, the MET activity of the three kinds of metal complex-containing polymers: PVI[Os(dmebpy)₂Cl] with neutral ligands, PVI[Os(dcbppy)₂Cl] and PVI[Fe(CN)₅] with negatively charged ligands (PVI = poly(1-vinylimidazole); dmebpy = 4,4'-dimethyl-2,2'-bipyridine; dcbppy = 4,4'-dicarboxy-2,2'-bipyridine), has been estimated for four H_2O_2 -producing flavoenzymes. The results reveal that the MET reactivity of the negatively charged polymers is very low, which is strongly related to the electrostatic repulsive interaction between the local surface charge of the flavoenzymes and the polymer's charge as well as the steric hindrance. Especially, in PVI[Fe(CN)₅], the polymerization of Fe(CN)₅ via PVI increases the negative charge density and then enhances the electrostatic repulsive effect. However, PVI[Fe(CN)₅] works as a good mediator for the POD reaction to reduce H_2O_2 . This specific catalytic property of PVI[Fe(CN)₅] is very convenient to construct H_2O_2 -producing oxidase/POD-based bienzyme biosensors.

For the application, in *Chapter 2*, PVI[Fe(CN)₅] has been utilized for constructing the creatinine biosensor which is based on the reductive H_2O_2 detection. PVI[Fe(CN)₅] is appropriate for the enzyme immobilization in this biosensing system and it shows the superiority of being a mediator, since it only mediates the POD reaction but not against the sarcosine oxidase reaction. The interference effect due to the poor specificity of

POD has been successfully eliminated by the Nafion film and the proposed method is applicable for clinical diagnosis.

To construct highly sensitive biosensors mediated by PVI[Fe(CN)₅], in *Chapter 3*, D-amino acid oxidase (DAAO)/POD bienzyme biosensor mediated by PVI[Fe(CN)₅] has successfully been constructed for the DAA determination. There is no cross reaction between PVI[Fe(CN)₅] and DAAO, and the severe interference effect resulted from high concentrations of interference has been eliminated by the Nafion films and AO. The stability of DAAO has been improved by adding free flavin adenine dinucleotide (FAD) and the Nafion film prevents the considerable leakage of FAD. The biosensor has low detection limit and high sensitivity, which indicates its applicability for clinical diagnosis. Since the main species of DAAs in human brain is D-serine, the proposed biosensor seems to have a potential for D-serine determination in brain without the problem of the DAA specificity of DAAO.

In summary, the author provides the information about the interaction between several enzymes and polymers in this research, and has successfully used the specific catalytic property of PVI[Fe(CN)₅] to overcome the problem of cross reaction in two kinds of bienzyme biosensors. The strategy in this research greatly improves the sensitivity and specificity of bienzyme biosensor, and the author hopes the achievement pushes the development of biosensor.

Appendix

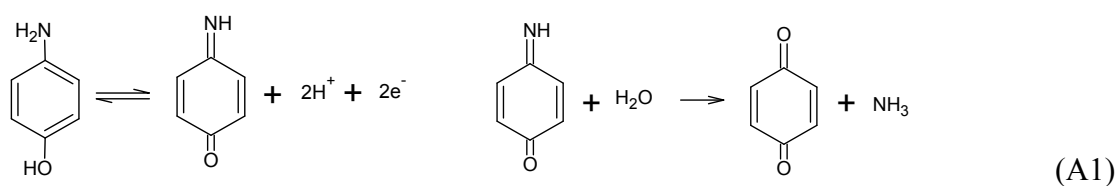
The reactions of several quinones/phenols with sarcosine

Several quinones/phenols were investigated to check the reaction with sarcosine. Some have been found to react with sarcosine directly: 1,4-benzoquinone (1,4-BQ), 2,5-dichloro-1,4-BQ, 2,6-dichloro-1,4-BQ, 2,6-dimethyl-1,4-BQ (all generate purple product); 9,10-phenanthrenequinone and 2,3,5,6-tetramethyl-1,4-BQ (both generate yellow-white product); 2,3,5,6-tetrachloro-1,4-BQ (it generates brown-green product).

CRH/SOD electrode was used for investigating the reaction between sarcosine and mediator, and between sarcosine and SOD. The cyclic voltammograms of CRH/SOD electrode with 2,6-dichloro-1,4-BQ, 1,4-BQ and 2,6-dimethyl-1,4-BQ addition in the creatine solution are shown in Fig. A1. All quinones work as mediators for SOD. Since the colored product was generated on the electrode surface, the catalytic oxidation current decreased with time. The change of cyclic voltammetric profile in the presence of 2,6-dimethyl-1,4-BQ (Fig. A1C) is hard to be observed since the generation rate of colored product is slow. The comparison of generation rate is shown as follows: 2,6-dichloro-1,4-BQ > 1,4-BQ > 2,6-dimethyl-1,4-BQ.

2,4-diaminophenol and *p*-aminophenol were also tested for SOD reaction by CRH/SOD electrode and the results show both react with SOD (Fig. A2). However, 2,4-diaminophenol is unstable in pH 8 buffer; the cyclic voltammetric profile of 2,4-diaminophenol shifted and the peak decreased with time (Fig. A2B), while *p*-aminophenol may react with water to produce 1,4-BQ (Eq. A1), which reacts with sarcosine.

Some show no mediating capability against SOD: 3,5-di-*tert*-butyl-1,2-BQ, 2,6-di-*tert*-butyl-1,4-BQ and 9,10-nathrenequinone.



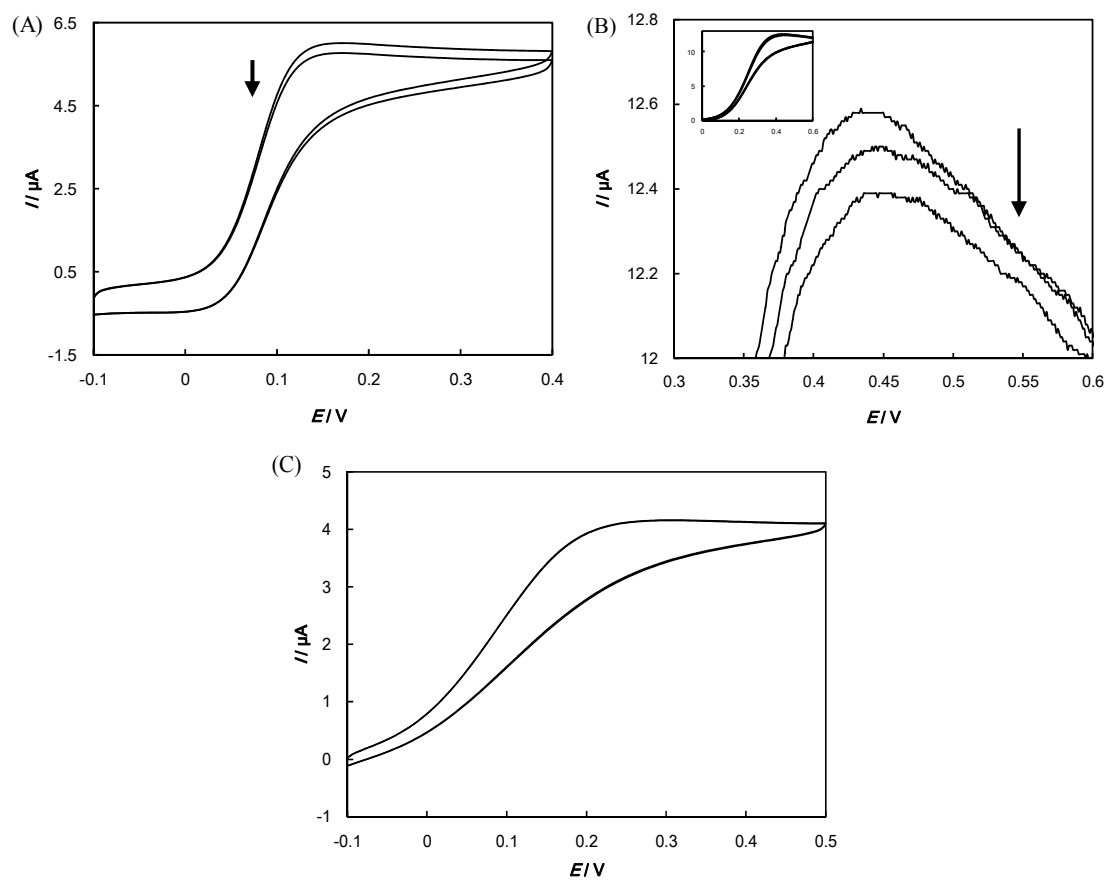


Figure A1 Cyclic voltammograms of CRH/SOD electrode in the pH 8, 50 mM creatine solution containing 250 μM (A) 2,6-dichloro-1,4-BQ, (B) 1,4-BQ and (C) 2,6-dimethyl-1,4-BQ. The arrows represent the successive scan. The CV in (B) is one part of the inset in (B). Enzymes were immobilized with glutaraldehyde. Scan rate: 20 mV s^{-1} .

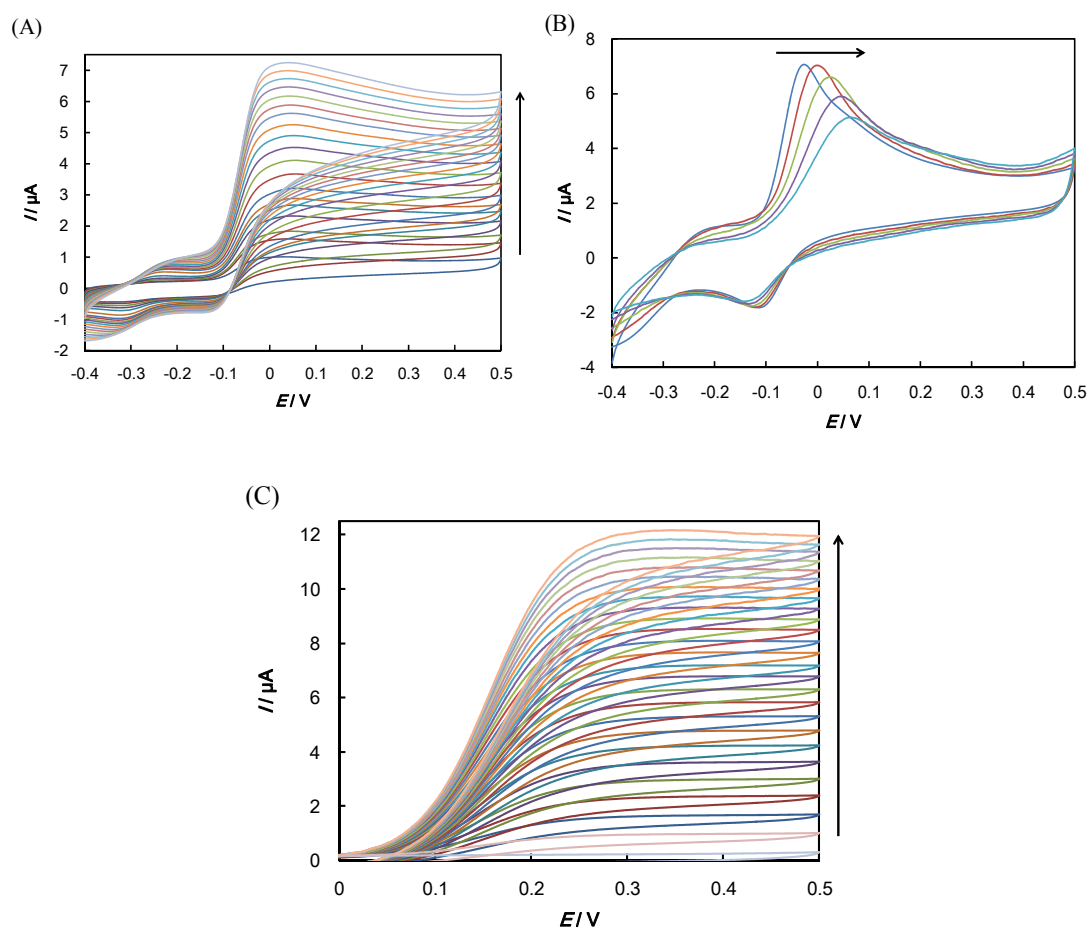


Figure A2 Cyclic voltammograms of (A) CRH/SOD electrode in pH 8, 50 mM creatine solution with successive addition of 10 μM 2,4-diaminophenol (arrow direction); (B) 0.5 mM 2,4-diaminophenol (pH 8). The arrow represents the successive scan; (C) CRH/SOD electrode in pH 8, 50 mM creatine solution with successive addition of 10 μM *p*-aminophenol (arrow direction). Scan rate: 20 mV s^{-1} .

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List of publications

- 1) Chi-Hua Nieh, Yuki Kitazumi, Osamu Shirai and Kenji Kano
Sensitive D-amino acid biosensor based on oxidase/peroxidase system mediated by pentacyanoferrate-bound polymer.
Biosens. Bioelectron. 47 (2013) 350-355. (*Chapter 3*)
- 2) Chi-Hua Nieh, Seiya Tsujimura, Osamu Shirai and Kenji Kano
Amperometric biosensor based on reductive H₂O₂ detection using pentacyanoferrate-bound polymer for creatinine determination.
Anal. Chim. Acta 767 (2013) 128-133. (*Chapter 2*)
- 3) Chi-Hua Nieh, Seiya Tsujimura, Osamu Shirai and Kenji Kano
Electrostatic and steric interaction between redox polymers and some flavoenzymes in mediated bioelectrocatalysis.
J. Electroanal. Chem. 689 (2013) 26-30. (*Chapter 1*)